

Thesis  
9.299

STUDIES ON THE REPRODUCTIVE BIOLOGY  
OF  
OREOCHROMIS NILOTICUS L.

A thesis presented for the degree of  
Doctor of Philosophy to the University of Stirling

by

PENPUN SRISAKULTIEW, B.Sc, M.Sc

Institute of Aquaculture  
University of Stirling

Scotland

U.K.

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## DECLARATION

This work has been composed entirely by my own investigation. Except where specifically acknowledgement work in this thesis has been conducted independently and has neither been accepted nor is being submitted for any other degree.

.....*P. Srisakultiew*..... Candidate  
.....*A. S. Bana*..... Supervisor  
.....*23 / 6 / 93*..... Date

## ACKNOWLEDGEMENTS

I am indebted to my supervisor, Dr. K.J. Rana, for his supervision during this work and his invaluable help in reading and correcting this thesis.

Special regard to Dr. Zvi Yaron, Tel Aviv University, Israel, for kindly analyzing the taGtH samples in experiment 4 of chapter 7.

I would also like to thank all my friends who have helped me throughout this work, in particular to Clive Randall & Briony Campbell for steroid radioimmunoassays and useful suggestions; Mark Thrush for statistical analysis; Ann Gilmore, my English teacher, for correcting and reading my thesis; Marguerite Mason for EM, film processing and printing techniques; Maureen Menzies for help in some of my works; Billy Struthers for help with the spectrophotometer; Keith Ranson and Willy Hamilton for system maintenance in the tropical aquarium; Brian Howie for all technical works; James Bron, my flat mate, for daily English and use of his laser printer for the printing of this thesis.

Nevertheless, I am specially grateful to my 'Scot's family', Betty Stenhouse and her family for all the support throughout my living here. Thanks to the Thai government for the opportunity to come here and a special sincere thank you to the British Council for the grant throughout my study and residence here.



**TO MY FAMILY**

## ABSTRACT

This study investigated the reproductive biology of *Oreochromis niloticus* broodstock of known age structure and spawning history with the aim of synchronising and controlling their spawning for mass fry production.

Hatchery reared stock was subjected to a constant photoperiod of 12L:12D and maintained at  $27 \pm 1^{\circ}\text{C}$ . All stock was fed on commercial trout pellets. The feeding frequency and protein content of the diet varied depending on fish size.

Oocyte development was classified into 6 stages including that of atresia based on histology. In order to quantify ovarian maturity, three stereological methods were compared. The ovarian volume fractions of different oocyte stages estimated by the mass, graphical and intersection methods showed homogeneous results. The intersection method required less time (2.6 mins/sample) whereas the others needed 11-12 mins/sample. In addition, the numerical density technique employing the intersection method was used and yielded similar oocyte estimates to those derived from the Gilson's fluid method.

Onset of sexual differentiation was influenced by the stocking densities. At 10 and 20 fry/l, 30 and 45% of those fry, respectively, were sexually differentiated by day 11 post-hatch, whereas those held at 2 fry/l were not.

Gonadal development was monitored in fish of known age. Fry were randomly sampled after hatching at two week intervals until 24 weeks. Total body length and weight were recorded and gonads were fixed for maturity determination. Serum samples were analyzed for total calcium ( $\text{Ca}^{2+}$ ), testosterone (T) and oestradiol-17 $\beta$  ( $\text{E}_2$ ). The males grew faster than the females of the same age and showed secondary sexual characteristics and attained maturity with significantly ( $P < 0.05$ ) higher T levels by 16 and 22 weeks, respectively. Females in comparison showed a significant ( $P < 0.05$ ) increase in GSI during 18 - 24 weeks (0.5 - 3.6%). The volume fraction of stage 6 oocytes, which were positively correlated to GSIs ( $r^2 = 0.84$ ;  $P < 0.05$ ), increased from 46.7% (20 weeks) to 71.8% by 22 weeks and then declined to 67.5% by 24 weeks. These results coincided with the mean levels of  $\text{E}_2$  whereas the  $\text{Ca}^{2+}$  and T levels showed high average levels through 24 weeks. These trials suggested that the females attained sexual maturity by 22 weeks.

Ovarian recrudescence and average levels of  $\text{Ca}^{2+}$ , T and  $\text{E}_2$  over 2 to 3 spawning cycles were studied. Within each spawning cycle the volume fraction of stage 6 oocytes increased from 0 - 15% (at day 1) to 65 - 72% by day 10 after spawning, which coincided with the high levels of  $\text{Ca}^{2+}$  and T whereas  $\text{E}_2$  levels peaked at day 5 and then decreased at day 10 after spawning. Females at day 10 post-spawning had, therefore, completed vitellogenesis and spawning occurred at the median time of 13 days.



In addition, average hormonal levels, egg quality and quantity over 2 to 3 spawning cycles were monitored in eight individual females. Females were bled twice a week after their first spawning. The median of spawning cycles of these females for the first and second cycles were 13 (short cycle) and 28 days (long cycle), respectively, and their overall median spawning cycle was 15 days (short cycle).

Levels of  $E_2$  were significantly ( $P < 0.05$ ;  $r^2 = 0.79$ ) correlated to the volume fractions of stage 6 oocytes and their peak levels were significantly correlated ( $P < 0.05$ ;  $r^2 = 0.49$ ) to fertilisation rates of eggs in subsequent spawns. Fecundity and fertilisation rates of eggs from those females in the second and third spawning were higher than the first spawning which indicated that the females that had spawned previously tend to ovulate more eggs than those that had spawned for the first time. The spawning history showed no effect on their fertilisation rates.

The females which were selected by their external characteristics were either injected (10 to 300  $\mu$ g D-Ala<sup>6</sup>-Gly<sup>10</sup>-LHRH + 0.1mg pimozone/kg body weight) or implanted (fast or slow release pellets containing LHRH; 100  $\mu$ g/kg) with the hormones. Neither the injections nor LHRH pellets were effective in inducing the females to spawn.

At day 10 after each spawning, a mixture of 100  $\mu$ g LHRH + 0.1mg pimozone/kg body weight was injected into the females kept under two spawning conditions. Females were held in

either separated compartments (limited contact) or under normal communal spawning conditions (unlimited contact). Spawning environment affected the success of induced spawning. The females which were held in the separated compartments spawned within 2 to 6 days post-injection whereas the sham controls spawned in 7 to 8 days post-injection. In contrast, the females in the communal spawning environment did not respond to hormone induction. The timing at day ten post-spawning and the conditions of spawning were found to be the important factors affecting exogenous hormonal administration in this fish species.

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## CHAPTER 1

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## 1. GENERAL INTRODUCTION

The Nile tilapia, *Oreochromis niloticus*, is a freshwater fish which belongs to family Cichlidae, subfamily Tilapiine (Trewavas, 1983). Due to its suitability for aquaculture, this species which originates in Africa, is now widely distributed for culture throughout many tropical and sub-tropical countries including Africa, America, Middle East and Asia (Pullin, 1983).

### 1.1 ROLE OF TILAPIAS IN AQUACULTURE

The tilapias are ideal fish for improving the nutrition and increasing the income of rural populations in many developing countries (Pruginin, 1983). In addition species such as, *O. niloticus* are excellent table fish, with firm white flesh and no intermuscular bones (Wohlfarth and Hulata, 1983). Therefore, they are a great potential species for commercial farming in developed countries such as USA.

*O. niloticus* is the most important tilapia species for aquaculture. It has a fast growth rate, is an omnivorous feeder, is tolerant to low dissolved oxygen, temperature changes and only stops reproducing at high salinities of 15-18 ppt (Pullin, 1983). They also consume a wide range of foods such as natural manure and artificial feeds and are

resistant to diseases, overcrowding and a wide range of water quality. These attributes can result in high yields, for example, *O. niloticus* cultured in ponds fertilized with only sewage for 5 to 7 months yielded 5 to 6 tons/ha/year (Edwards, 1988); and *O. andersonii* in an integrated pig farm yielded 4 to 6 ton/ha/year (Gopalakrishnan, 1988).

## 1.2 PROBLEMS OF TILAPIAS IN AQUACULTURE

The major drawback with tilapia is their ability to spawn at as young an age as 4 to 5 months (Wohlfarth and Hulata, 1983) and at a size as small as 6 - 15cm (Babiker and Ibrahim, 1979a; Dadzie and Wangila; 1980). Due to this spawning ability the ongrowing ponds are rapidly overcrowded with a mixed size range of fish and at harvest only a small proportion of fish may reach marketable size (200-250g).

## 1.3 METHODOLOGIES FOR TILAPIA CULTURE

To overcome this unwanted reproduction, several techniques have been tried to control reproduction in grow out ponds. Polyculture with predators such as *Cichla ocellaris* (Verani, Pinto, Paiva and Tabata, 1983), *Lates niloticus* (Ofori, 1988) and *Penaeus monodon* (Gonzales-Corre, 1988), high stocking density (30 fish/m<sup>2</sup>) in intensive culture (Pruginin, Fishelson and Koren, 1988) and finally monosex (all male) cultures (Mires, 1977; Sin and Chiu, 1983; Verani



et al., 1983 and Hanson, Smitherman, Shelton and Dunham, 1983) have been successfully applied to control production of these fish.

As the male tilapias grow faster than the females and the culture of a single sex culture eliminates propagation of the fish, the use of monosex culture of males is the most favoured method of tilapia culture (Mires, 1977; Guerrero, 1982; Hanson et al., 1983). All male tilapia can be obtained using various methods. The males can be graded by hand or mechanical graders using growth or size differences (Guerrero, 1982), or by hand sexing of 50 g fish by examining the urogenital papillae (Mires, 1977; Guerrero, 1982). Hybridization between different tilapia species such as *Oreochromis nigra* x *O. hornorum* and *O. niloticus* x *O. variabilis* can also be used to produce 100% males (Mires, 1977). Alternatively, sex-reversal techniques using male steroid hormone can be used to produce all male fry (Hanson et al., 1983; Mires, 1983).

For mass fry production, however, all these techniques require large numbers of fry of the same age or size. Unfortunately, the mass production of tilapia fry is made difficult by their unpredictable spawning and breeding patterns. The asynchronous spawning of females often requires large pond areas for seed production and requires the management of large numbers of broodstock.

#### 1.4 FECUNDITY OF TILAPIAS

Fecundity in tilapias has been defined in various ways. Lowe-McConnell (1955) defined fecundity as the numbers of fry produced in the lifetime of an individual fish. Mires (1982) reduced the lifetime fecundity to a 12 month period starting from the first spawning. Alternatively, size distribution of oocytes in pre-spawning females can be used as a basis to estimate fecundity. As tilapias are multiple spawners; their ovaries show a bimodal oocyte size distribution. This distribution, however, does not consider the level of atresia prior to spawning, residual eggs after spawning or eaten or swallowed during buccal rearing (Peter, 1983; Rana, 1986). Therefore, the fecundity of tilapias should be defined as the number of eggs in a freshly spawned clutch (Rana, 1988).

*O. niloticus* females produce relatively few large eggs at each spawning. Although their egg numbers are small, hatching and survival rates of eggs or fry are high (Lowe-McConnell, 1959; Peter, 1983; Rana, 1988). For example, the females in this species were reported to produce approximately 700 to 2,000 eggs/spawn and 80 to 95% egg survival rates (Wohlfarth and Hulata, 1983; Rana, 1986). The size of tilapia eggs is not constant, larger eggs are produced from larger females (Lowe-McConnell, 1959; Welcomme, 1967; Trewavas, 1983; Rana, 1986). The egg size, however, is inversely related to clutch size; thus either



large number of small eggs or small numbers of larger eggs are produced by the fish (Peter, 1983; Rana, 1988). For example, small *O. niloticus* females (16 - 71g) produced small eggs (mean = 4mg) approximately 13,168 eggs/kg whereas bigger *O. niloticus* (180 - 489g) released large eggs (mean = 7.8mg) about 3,339 eggs/kg (Siraj, Smitherman, Castillo-Gallusser and Dunham, 1983; Rana, 1988).

### 1.5 SPAWNING FREQUENCY AND SPAWNING CYCLES OF TILAPIAS

Peter (1983) reported that wild tilapias spawned at least twice a year. Detailed information on the spawning frequency of wild species, however, is difficult to ascertain. In captivity, Fishelson (1966) reported 11 clutches of eggs in a year from a single female robbed of her clutch after spawning and maintained under optimum spawning temperature. In contrast, Mires (1982) reported that *O. niloticus* females spawned only 2 to 7 times/year at average breeding cycles of 23 to 50 days when maintained under similar spawning condition as Fishelson (1966).

Using hapas suspended in a concrete tank and collecting the *O. niloticus* fry at three days after mouthbrooding, Siraj et al., (1983) reported spawning cycles of 7 and 12 days for one and two year old females, respectively. In comparison, Rothbard, Solnik, Shabbath, Amado and Grabie (1983) presumed the spawning cycle of hybrid tilapia (*O. niloticus* x *O. aureus*) in earthen pond to be 13 to 18 days.

In conclusion, the spawning cycle or spawning frequency of tilapias is still unclear. To gain a better understanding and predictability of spawning in tilapias, various factors which control and trigger ovarian maturation and spawning will need to be considered.

## 1.6 FACTORS CONTROLLING TELEOST REPRODUCTION

### 1.6.1 Exogenous Factors

The events of teleost reproduction are controlled by a series of interrelated external and internal stimuli. Fish respond to changes in external conditions such as day-length, temperature, spawning substrate, social factors, food availability, water quality, etc. These changes are then translated as neural inputs which subsequently stimulate the brain and gonads to respond to the above triggers (De Vlaming, 1972; Stacey, 1984).

#### 1.6.1.1. Photoperiod

Photoperiod has been used to manipulate the timing of spawning in several teleost species. In goldfish, *Carassius auratus*, for example, when the fish were maintained at 16L:8D (20°C), gonadotropin (GtH) levels increased during the latter half of the photophase and peaked during the latter half of scotophase, during which time follicular rupture occurred. The levels of GtH? were then peaked and

spawning of the goldfish commenced after the following photophase (Stacey and Peter, 1979). In medaka, *Oryzias latipes*, ovulation also occurs during the latter portion of scotophase and almost every day of the breeding season. Ovulation and oviposition, are triggered by the synchronous release of GtH under appropriate photoperiod (Iwamatsu, 1978).

In salmonids, spawning appears to rely on the seasonally changing cycle of daylength in relation to the time of gonadal recrudescence and maturation (de Vlaming, 1972). Spawning of these fish under constant 6L:18D or 12L:12D photoperiods occurs at about yearly intervals (Whitehead, Bromage, Forster and Matty, 1978b; Bromage and Duston, 1986; Duston and Bromage, 1986).

Photoperiod has been reported as an effective, convenient and a relatively cheap method for the manipulation of spawning time. This method requires light-proof material to cover tanks or ponds, tungsten bulbs for illumination of at least 40 lux at the water surface and an electronic timer to control the required photoperiod regime (Bromage and Cumaranatunga, 1988).

#### 1.6.1.2. Temperature

Temperature, influences and plays an important role in many hormone functions in teleost reproduction. The increase



and/or decrease in hormone responses, gonadal binding with hormones and daily cycles, synthesis and catabolism of steroids and GtHs all respond to temperature fluctuations (Hontela and Peter, 1978 and Stacey, 1984).

In goldfish maintained under natural photoperiod and at temperatures below 14°C, ovaries are arrested at the end of exogenous vitellogenesis. Ovulation can be induced at any time of the year when the temperature increases over 20°C (Yamamoto, 1969).

In tilapia testes, spermatogenesis occurs all the year round even at temperatures as low as 16°C (Hyder, 1969) and the male fish show breeding behaviour when the temperature increases above 20°C (Fishelson, 1966).

Oogenesis in tilapia ovaries is stimulated when the water temperature increases above 22°C (Wohlfarth and Hulata, 1983) and optimum spawning temperatures are reported to be 25 - 29°C (Rothbard and Pruginin, 1975). Rapid change in temperatures may also trigger endocrine secretions (Stacey, 1984). For example, when mature *O. niloticus* maintained at 29° - 32°C were suddenly moved to cold water (22 - 25°C) for 6 hours and transferred back to the previous temperature, the proportion of cold treated female spawning increased from 10 to 25% (Srisakultiew and Wee, 1988).



In cyprinids, low water temperature inhibits ovulation (Bye, 1984). Sexual development and spawning of cyprinids is modulated by both temperature and photoperiod. (Billard and Breton, 1981). Cyprinids normally spawn in spring and in summer. Gametogenesis of the spent fish may commence immediately and may be completed by autumn (October). Over this period the developing gametes are arrested until the temperature increases for final maturation and ovulation in the following season. In tench, *Tinca tinca*, elevated temperatures also accelerate gonadal maturation and prolong the spawning season. Gametogenesis is blocked at temperatures below 10°C in winter and resumes in spring (Billard, Breton, Fostier, Jalabert and Weil, 1978).

In some species elevated temperatures can retard gametogenesis. For example, in the cyprinid, *Mirogrex terrae-santae*, high temperatures (27°C) inhibit vitellogenesis but the cooler temperatures of the winter months stimulate ovarian recrudescence. The cooler temperatures enhance the production of vitellogenin in the liver in response to oestrogen stimulation (Yaron, Cocos and Salzer, 1980).

Combinations of photoperiod and temperatures can be used to manipulate the fish to spawn. For example, in salmonids, decreasing the photoperiod following the summer months induces the production of sex steroids and recrudescence of oocytes, and further stimulation of constant short

daylength triggers the ovaries to mature (Whitehead et al., 1978; Billard et al., 1978; Bromage and Cumaranatunga, 1988). Thus, it is possible to provide rainbow trout eggs and fry outside the natural spawning season. The spawning season has been accelerated and extended with photoperiod and temperature. Such manipulations result in the control of the variety strains of rainbow trout to spawn in every month of the year (Bromage, 1982).

#### 1.6.1.3. Spawning substrate

Under captive conditions, many teleosts complete vitellogenesis but fail to ovulate. This may indicate a lack of appropriate exogenous stimulation which is required for ovulation. In goldfish and common carp, for example, the presence of spawning substrate can induce spawning. Studies by Stacey and Peter (1979) suggested that the absence of appropriate substrate such as aquatic vegetation inhibits ovulation.

In nature, tilapias construct circular depressions in clay soils with a diameter of up to 1m and a depth of 0.5 m, or at a diameter of about twice of the male length (McKaye, 1984). In captivity, many tilapia species are able to spawn without clay soil-spawning nests (Lowe-McConnell, 1959; Fishelson, 1966; Rothbard, 1979) or other substrates. This may suggest that the spawning mechanisms of these tilapias are insensitive to spawning substrates (Stacey, 1984).



#### 1.6.1.4. Social factors of visualization and pheromones

In some teleost species, visual and chemical (pheromones) stimuli can increase the spawning frequency. For example, the stimulation of females by territorial males may influence the occurrence of ovulation in blue gourami, *Trichogaster trichopterus* (Liley, 1982).

Pheromones are a group of chemical substances emitted by an organism into the environment as a specific signal to other organisms, usually of the same species. The pheromones play an important role in the social behaviour of certain animals, notably mammals and insects. They are used to attract mates, to mark trails and to promote social cohesion and reproduction of teleosts (Stacey, 1987, 1989; Van Weerd, 1990). The pheromones affecting reproduction of fish are called sex-pheromones. These sex-pheromones regulate the spawning behaviour and endocrine events. For example, the sex-pheromones from ovulated zebrafish, *Brachydanio rerio*, attract the males and vice versa. In goby, *Gobius jozo*, and African catfish, *Clarius gariepinus*, the male sex-pheromone also attract the females (Van Weerd, 1990). Sex-pheromones are reported to influence oocyte maturation and ovulation in females by increasing the levels of gonadotropin (GtH) (Stacey, Sorensen, Van Der Kraak and Dulka, 1989) and stimulating post-ovulatory ovarian development in the tilapia, *O. mossambicus* (Silverman, 1978a,b).

In conclusion, a variety of exogenous factors may activate or inhibit the reproductive cycle. Temperature and photoperiod may also interact with endogenous cycles of teleost to ensure that the gonads mature at the appropriate time, but the ovulation and spermiation of gametes and spawning event may require other stimuli (Stacey, 1984).

It is possible that there is a minimum period required for full ovarian development. In the salmonids and flatfish, these periods may be around five to seven months, and may be related to the considerable energy investment of the ovary which may ultimately comprise 25 - 35% of the total fish weight (Bye, 1984).

In addition, gametogenesis may be influenced by sex, age, reproductive history and genetic strain. For example, when autumn and winter spawning strains of rainbow trout were subjected to the same condition of 18 hour daylength in March, gametogenesis in the autumn trout strain was delayed whereas that of the winter trout strain was advanced (Scott, Sumpter and Hardiman, 1983).

#### **1.6.2 Endogenous Factors**

It is widely reported that the fish integrate their physiological functions with exogenous stimuli (Fontaine, 1976; Sundararaj, 1981). Certain proximate environmental factors stimulate the pituitary via the central nervous



system (Figure 1.1). These endogenous rhythms which are synchronized at spawning time have been reviewed by de Vlaming (1972), Billard et al., (1978), Sundararaj (1981), Crim (1982) and Bromage and Cummaranatunga (1988). In females, when the endogenous factors are initiated by the appropriate exogenous factors, they mediate oogenesis. Previtellogenic oocytes develop into vitellogenesis, maturation and then ovulation (Stacey, 1984).

Endogenous levels of gonadotropins (GtHs), vitellogenin (Vg) or total calcium [ $\text{Ca}^{2+}$ ], which represents vitellogenin, and sex steroids (e.g., oestradiol- $17\beta$  [ $\text{E}_2$ ], testosterone [T],  $17\alpha$  hydroxy- $20\beta$ -dihydroxyprogesterone [ $17\alpha,20\beta$ -P], etc) in blood circulation of teleosts, are sensitive indicators in determining gonadal development. In rainbow trout, when these hormones increase with increasing daylength in spring, oocytes proceed from previtellogenic into vitellogenic stages (Figure 1.1; Bromage, Whitehead and Breton, 1982, Bromage and Cummaranatunga, 1988; Whitehead et al., 1983; Scott et al., 1983; Kobayashi et al., 1986; 1988; 1989).

The major endogenous hormones (Figure 1.1) involving teleost reproduction, include the hypothalamic gonadotropin-releasing hormone (GnRH), preoptic area gonadotropin release-inhibiting factor (GRIF), pituitary gonadotropin (GtH), steroids and prostaglandins. The increase of GtH prior to ovulation has been demonstrated in

several fish species. Details of the major endogenous hormones are described below.

#### 1.6.2.1. Gonadotropin releasing and inhibiting factors

King and Millar (1979; 1990) reported that the structure of teleost gonadotropin-releasing hormone (GnRH) is similar but not identical to the GnRH of reptiles and birds. The GnRH is secreted from the hypothalamus to stimulate the release of GtH from the pituitary gland (Figure 1.1).

When using brain lesions to demonstrate and identify sites of GnRH production, the lesions in the hypothalamic nucleus lateralis (NLT) resulted in a decrease of the gonadosomatic index (GSIs), e.g., male and female goldfish (Peter, 1970), male *Fundulus heteroclitus* (Pickford, Rea Knight, Knight, Gallardo and Baker, 1981) and Atlantic salmon parr (Dodd, Stuart-Kregor, Sumpter, Crim and Peter, 1978). Peter (1982) reported that in goldfish the NLT lesions also abolished their daily cycles of GtHs. These GtH cycles are necessary for ovarian recrudescence (Figure 1.1). In addition, the use of L-glutamate in goldfish to destroy neuronal perikarya provided evidence to support the role of NLT in GnRH regulation (Peter, 1980).

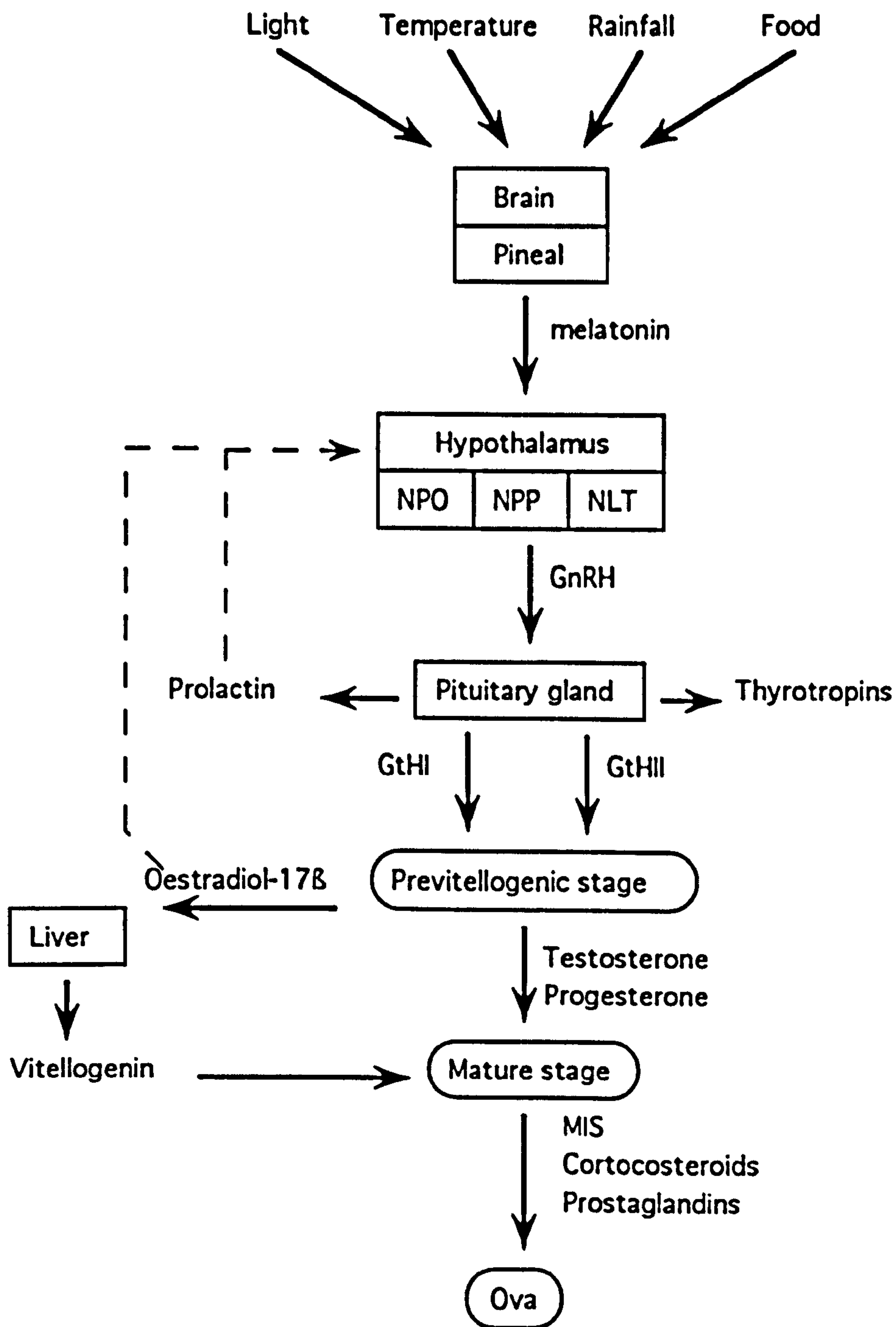


Figure 1.1: Schematic diagram of the principal components of mechanisms controlling oocyte development of teleosts. NPO = nucleus preopticus; NPP = nucleus preopticus periventricularis; NLT = nucleus lateral tuberis; GnRH = gonadotropin-releasing hormone; GtHs = gonadotropins; MIS = maturation inducing steroids.

Solid lines indicate stimulation, dash lines represent inhibition.



Based on these lesion studies in goldfish, Peter and Paulencu (1980) concluded that there was a gonadotropin releasing-inhibiting factor (GRIF). Lesions at the anterior of the preoptic brain area (source of GRIF) caused a dramatic increase of blood GtH which was followed by ovulation. Subsequent work strongly indicated that dopamine may function as a GRIF in goldfish. Blood GtH increased following an injection of either 6-hydroxydopamine (a catecholaminergic neurotoxin) or pimozide, a dopamine antagonist (Crim, 1982). These findings were consistent with an *in vitro* study in rainbow trout which showed that dopamine inhibited the release of GtH (Crim, 1982).

#### 1.6.2.2. Gonadotropins (GtHs)

Gonadotropins are secreted from the pituitary gland. Idler, Bazar and Hwang (1975); Idler and Ng (1979); Ng and Idler (1979) reported that there were two types of gonadotropins (see Figure 1.1). The first GtH (GtHI) has a low carbohydrate content, which stimulates vitellogenin uptake in oocytes. The second GtH (GtHII) has a high carbohydrate content, which stimulates oestrogen (vitellogenin synthesis), final maturation and ovulation. Recent studies on the physico-chemical characterization of gonadotropins in salmonids (Kawauchi, Suzuki, Itoh, Swanson and Nagahama; 1987; Suzuki, Kawauchi and Nagahama; 1987) revealed that GtHI and GtHII in the pituitary gland and serum were homologous to mammalian luteinizing hormone (LH) and

follicle-stimulating hormone (FSH) and they are differentially synthesized and released during reproductive development. Secretions of GtHI and GtHII depended on reproductive state of fish (Kawauchi et al., 1987; Suzuki et al., 1987). During early vitellogenesis, GtHI was found to predominate, whereas GtHII was higher than GtHI in pre-ovulatory and spermiating salmon (Kawauchi et al., 1987; Suzuki, et al., 1987). The potencies of GtHs in stimulating oestradiol-17 $\beta$  production were similar. GtHII, however, was more potent than GtHI in stimulating maturational steroid (17 $\alpha$ ,20 $\beta$ -diOHpreg) production in the thecal layer and in activating 20 $\beta$ -HSD in the granulosa layer (Hirose, 1976; Jalabert, 1976; Swanson, 1987).

In mammals, secretion of GtHs depends on GnRH levels and frequency of GnRH stimulation and physiological states of animal reproductive system. When GnRH $\alpha$  was applied to fish at different reproductive states, the fish responded to the GnRH differently. For example, when GnRH stimulated the fish at pre-vitellogenic stage, the fish secreted mostly GtHI. In contrast, when the GnRH stimulated fish in pre-ovulatory phase, the fish released more GtHII than GtHI (Kawauchi et al., 1987; Suzuki et al., 1987).

Endocrine rhythms associated with fish reproductive cycles, can be divided into long and short-term patterns of hormonal changes (Crim, 1982). The long-term pattern is a pattern that shows several spawning cycles within a year or



a breeding season. In seasonal breeders, the gonadosomatic index (GSI) or gonad weight is often used to designate progressive reproductive development. The GSI increases in response to the pre-spawning period and falls to minimum during the post-spawning period. Pituitary and plasma gonadotropin (GtH) levels also fluctuate during the year and peaks at the time of spawning. In precocious male Atlantic salmon parr, the pituitary GtH levels were maintained at low levels during their onset of testicular growth but then rapidly increased at the time of spawning (Crim and Evans, 1978). Similar cycles for pituitary GtH have been described in cyprinids, rainbow trout and brown trout (Billard et al., 1978).

The short-term pattern is that which shows a 24 hour rhythm of pituitary GtH levels. In goldfish, Hontela and Peter (1978) described daily serum GtH levels of females at different stages of oocyte development. These daily surges of high GtH appeared in the serum of vitellogenic and mature female goldfish but small peaks of daily GtH also occurred in the serum of sexually regressed females. Hontela and Peter (1978) concluded that the daily GtH surges are of physiological importance with respect to the process of gonadal recrudescence.

This may explain the absence of GtH progression in some teleosts during the period of gonad growth which results in the fish failing to spawn (Peter and Crim, 1978). The gonad

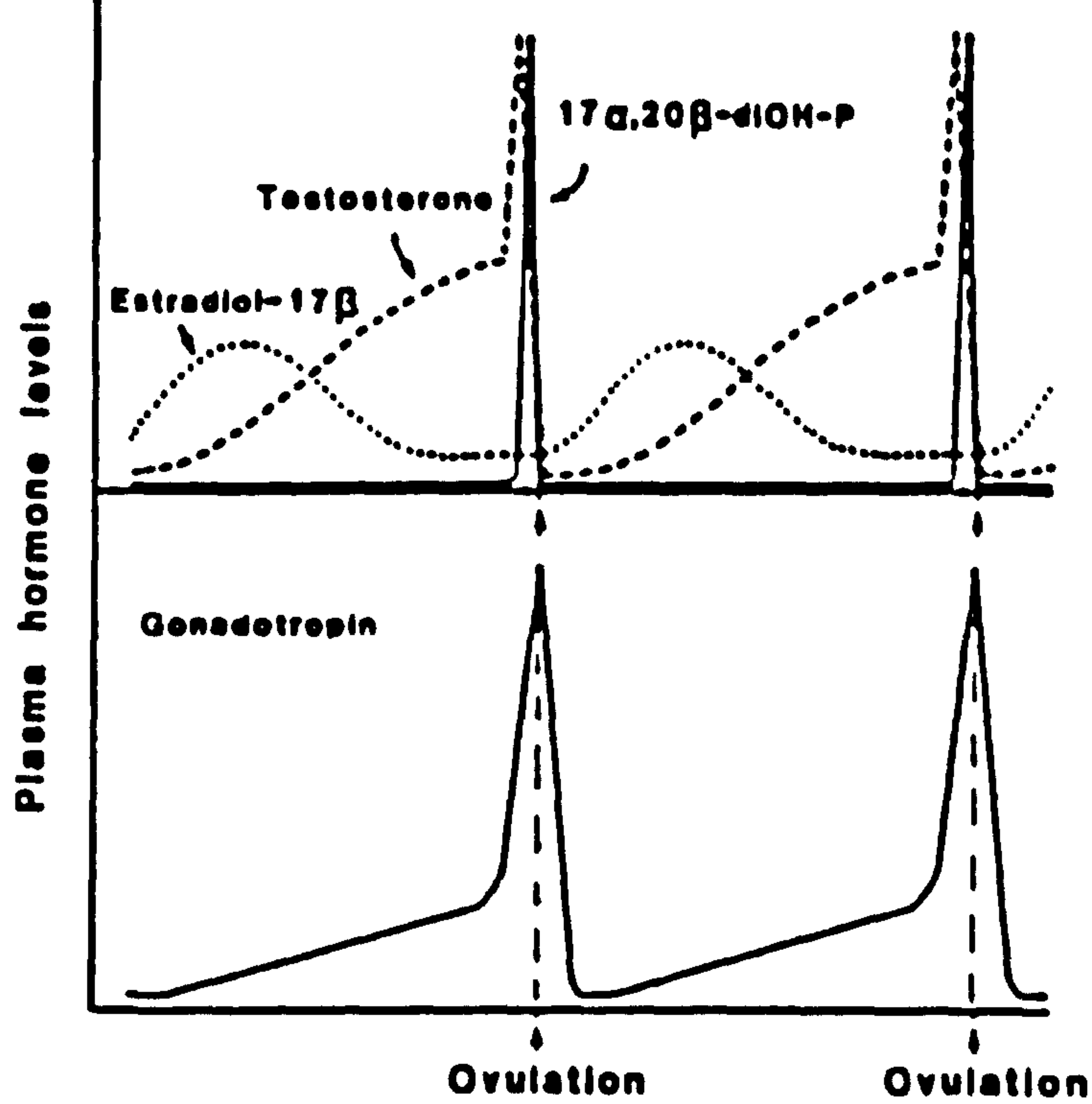


responsiveness to GtHs also varies with gonadal development (Crim and Evans, 1983). In catfish, a period of oocyte growth is followed by a resting period of the yolky oocytes during their reproductive cycle (Sundararaj and Anand, 1972). High GtH levels were found to be essential for maintaining maturation of yolky oocytes in gravid catfish but greater quantities are required during vitellogenesis and ovulation (Figure 1.2). The temporary responsiveness of gonads to hormone stimulation may result from daily GtH fluctuations. In female gold fish, a long photoperiod with warm temperature provides the optimum conditions for daily surges of GtH and for recrudescence (Hontela and Peter, 1978). These results suggest that the phasing of individual endocrine variation could be of importance and the synchrony of endocrine surges or the lack of it should be considered.

#### 1.6.2.3. Steroids

Steroid hormones are mediated by GtH action on oocyte maturation (see Figure 1.2) and hydration at the final stage of spawning. Steroid biosynthesis was reported to occur initially in the thecal layer of the developing follicles. It is transferred to the granulosa layer where the steroids are converted to oestrone, oestradiol-17 $\beta$  or 17 $\alpha$ , 20 $\beta$ -dihydroxy-4-pregnen-3-one (17 $\alpha$ ,20 $\beta$ -P; Kagawa, Young, Adachi and Nagahama, 1982; Nagahama, 1983). The levels of these steroids correspond with gametogenesis of

(a)



(b)

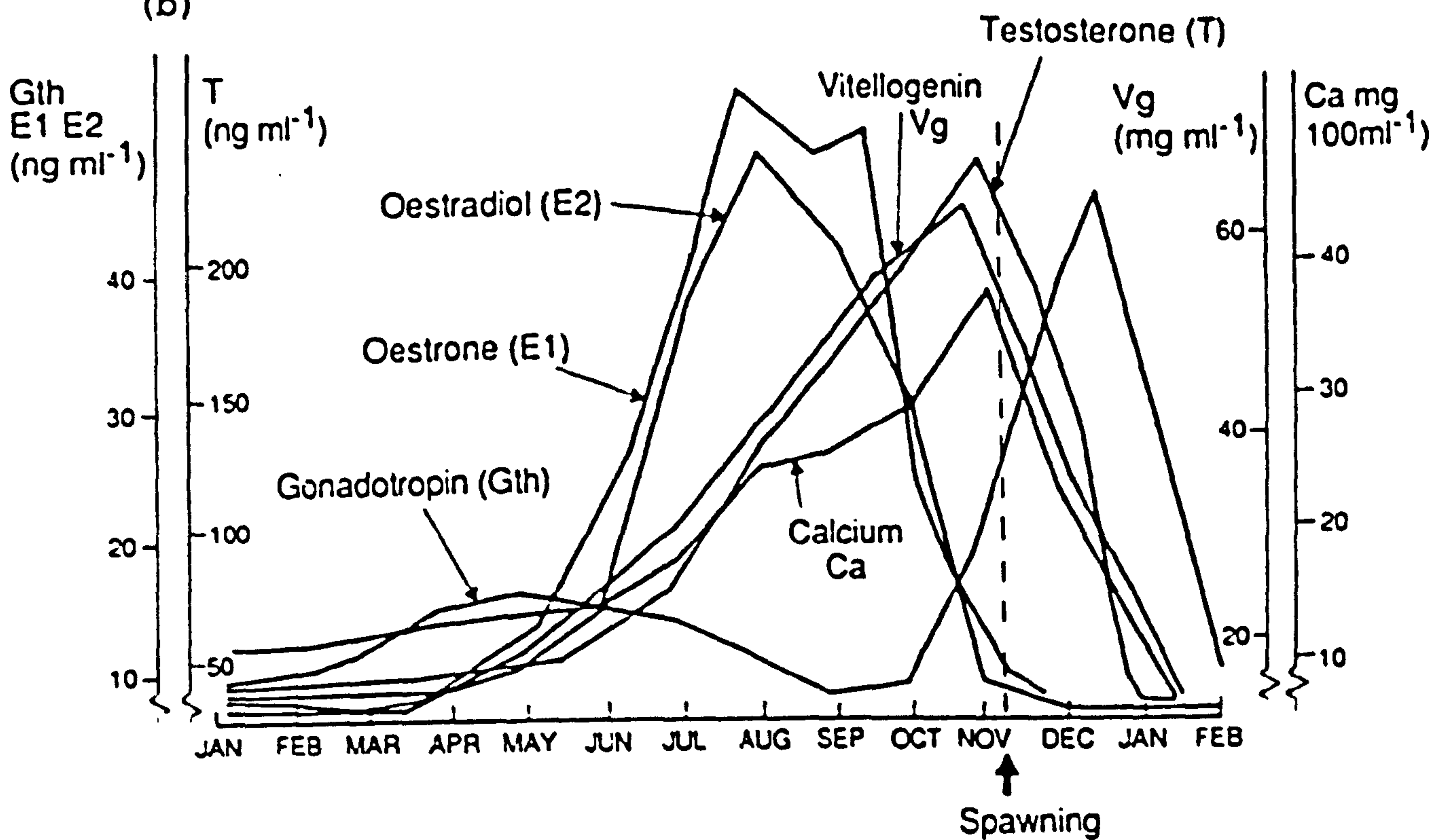


Figure 1.2: Profiles of various serum hormones during the annual reproductive cycle of: (a) goldfish (redraw from Kobayashi et al., 1988; 1989). (b) rainbow trout (from Bromage and Cumaranatunga, 1988). Arrow indicates time of ovulation.

teleosts. Changes in serum gonadotropin levels during the early ovarian development correspond to the increase of oestradiol-17 $\beta$ , oestrone, testosterone and vitellogenin (Figure 1.2; Whitehead et al., 1978; Scott et al., 1980; Bromage and Cumaranatunga, 1988; Kobayashi, Katsumi and Hanyu, 1988). Serum calcium ( $\text{Ca}^{2+}$ ) levels are correlated with vitellogenin levels (Figure 1.2). Since  $\text{Ca}^{2+}$  is easy to analyze at low cost, their levels have been adopted as an indicator of blood vitellogenin (Elliott, Bromage and Springate, 1984).

Referring to the increase in sex steroids e.g., in the migratory salmon, winter flounder, plaice and rainbow trout, it was found that all these steroid levels reach their peaks at spawning and then declined rapidly (Whitehead et al., 1978; Scott, Bye and Baynes, 1980; Idler, Horne and Sangalang, 1971; Campbell, Walsh and Idler, 1976; Wingfield and Grimn, 1977).

The steroid hormone called the maturation inducing steroid (MIS; see Figure 1.1; 1.2) or 17 $\alpha$ , 20 $\beta$ -P is reported to be implicated in final ovulation (Suzuki, Tamaoki and Hirose, 1981). Injection of this hormone led to germinal vesicle migration and its subsequent breakdown (Goetz, 1983). This steroid hormone is found in many teleosts, for example, rainbow trout, *Salmo gairdneri*, goldfish, *Carassius auratus*, northern pike, *Esox lucius* (Jalabert, 1976), yellow perch, *Perca flavescens* (Goetz and Theofan, 1979),



amago salmon, *Oncorhynchus rhodurus* (Nagahama, Kagawa and Tashiro, 1980) and Ayu, *Plecoglossus altivelis* (Suzuki et al., 1981).

#### 1.6.2.4. Prostaglandins

In teleosts, prostaglandins have been observed to stimulate mature oocytes to undergo final ovulation by the rupture and expulsion of the ova (Stacey and Goetz, 1982; Goetz, 1983).

*In vitro* studies in goldfish, using prostaglandin synthesis inhibitors (indomethacin) had shown that these inhibitors block follicular rupture but, the fish could be induced to spawn again with human chorionic gonadotropin (HCG) injection (Stacey and Pandey, 1975).

### 1.7 OBJECTIVES OF THIS THESIS

The main objectives of this study were to obtain a better understanding of the reproductive cycles of *Oreochromis niloticus* and eventually aim to synchronize spawning of the females for mass fry production.

There are several studies reporting the reproductive biology of *O. niloticus*. Most of these studies, however, relate to wild populations of either unknown age or spawning history. The present study was carried out on fish

reared in captivity and of known age structure, growing conditions and spawning history. The following trials were conducted to complete the main objectives of this thesis:

1. Classify oocyte development using histological techniques and to develop a more comprehensive classification of oocyte development which included atresia.
2. Evaluate methods for assessing oocyte numbers and quantify the volume fraction of the different oocyte stages in tilapia ovary and then select the most suitable method to estimate maturity of the fish.
3. Determine the onset of sexual differentiation, gonadal development and hormonal maturity in known age groups of *O. niloticus* during puberty.
4. Demonstrate ovarian recrudescence, hormonal profiles and spawning cycles of females at different periods after spawning.
5. Control the spawning of females by the application of the hormone, D-Ala<sup>6</sup>-Gly<sup>10</sup>-LHRHa by either injection or hormone releasing pellets.
6. Investigate the effect of breeding environment on induced spawning.

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## CHAPTER 2

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## 2. GENERAL MATERIALS AND METHODS

General details of materials and methods used throughout the studies for fish maintenance, histological studies, electron microscopical study, oocyte volume fraction estimation and blood sample analysis are included here.

### 2.1 FISH MAINTENANCE, HANDLING AND OVARIAN AND BLOOD SAMPLING

#### 2.1.1 Fish Maintenance

The Nile tilapia, *Oreochromis niloticus* used throughout this study was obtained from genetically pure broodstock, held at the Tropical Aquarium of the Institute of Aquaculture, Stirling University. The fish were stocked at 0.1-1 fish/l (depending on fish size) in 1 m<sup>2</sup> fibre glass tanks which were linked to a recirculating system. The water temperature was maintained at 27±1°C and the fish subjected to a photoregime of 12L:12D. The biological filters were cleaned twice a month and the fish were fed daily on a commercial trout feed (B.P Nutrition (U.K) Ltd., and Ewos Ltd., Bathgate, Scotland), either No. 3 (54% protein), No. 4 (40-42% protein) or No. 5 (40-42% protein).

### **2.1.2 Anaesthesia**

In order to minimise handling stress, fish were anaesthetized in 2-phenoxy ethanol (Sigma Chemicals, Dorset, England) at a dilution of 1:20,000 in water prior to tagging, weighing, bleeding, injecting or hormone implanting. After completing the various handling tasks, the fish were transferred into clean aerated freshwater where they recovered within 1-2 minutes.

### **2.1.3 Blood Sampling**

#### **2.1.3.1 Procedure for collecting serum**

Blood samples were taken from the caudal vein by using either 23G or 25G sterile hypodermic needles connected to sterilised 1 ml syringes (Terumo Europe N.V., Leuven, Belgium). The sampled blood was transferred into an eppendorf tube (Sarstedt Ltd. Leicester, U.K.), allowed to clot on ice and then centrifuged at 13,000 rpm for 5-10 minutes. The serum samples (supernatant) were removed with pipettes and transferred into new eppendorf tubes. The serum samples were then stored at -20° C until required for future analysis.

#### 2.1.3.2 Procedure for collecting plasma

Blood samples were taken from the caudal vein with an ammonium heparin-coated syringes connected to 23G hypodermic needles. The sampled blood was transferred into an eppendorf tube on ice and centrifuged at 2,500 rpm for 10 minutes at 4°C. The supernatant was removed, transferred into a new eppendorf tube and stored at -20°C until required for future analyses.

#### 2.1.4 Ovarian and Liver Sampling

Ovaries and livers were dissected from sacrificed fish. The left and right ovaries, livers and carcasses of individual fish were weighed separately. Gonadosomatic (GSI) and hepatosomatic indices (HSI) were determined as shown in the following formulas:

$$\text{GSI (\%)} = \frac{(L+R)}{C} \times 100$$

$$\text{HSI (\%)} = \frac{H}{C} \times 100$$

where

|   |   |                           |
|---|---|---------------------------|
| L | = | left ovary weight (g)     |
| R | = | right ovary weight (g)    |
| C | = | gutted carcass weight (g) |
| H | = | liver weight (g)          |



## 2.2 MORPHOLOGICAL STUDIES

For ovarian morphological studies, two 5-7 mm sections were dissected from the middle part of the ovaries and then fixed in Bouin's solution (Appendix 1.1) for light microscopical studies. For electron microscopical studies, 10 to 20 oocytes were dissected and immediately fixed in 2.5% glutaraldehyde.

### 2.2.1 Histological Studies

Preliminary studies (Cummaranatunga, 1985; Coello, 1989) indicated that infiltration of paraffin wax into advanced oocytes was minimal. As a result, the paraffin wax embedding process was unsatisfactory, resulting in large holes in the cut sections. Therefore, to improve infiltration, the resin technique was used as the histological embedding medium and applied to advanced ovaries. The paraffin wax embedding technique was restricted to immature ovaries and testes.

#### 2.2.1.1 Embedding techniques

After the tissue samples were fixed in Bouin's solution for 12-24 hours, they were rinsed and stored in 70% ethanol until required for either paraffin wax or resin embedding.

#### **a. Paraffin wax embedding**

Methods for paraffin wax embedding and staining were carried out according to the routine procedure used at the Histopathology Laboratory, Institute of Aquaculture, Stirling University. These methods were modified from Carlton's Histological Technique (Drury and Wallington, 1980).

The samples, fixed in Bouin's solution and kept in 70% ethanol, were dehydrated in a series of alcohols, finishing with 100% alcohol and cleared in chloroform in an automated tissue processor (Histokinette 2000) at the appropriate time intervals (Appendix 1.2.1). The paraffin wax impregnated samples were then blocked with wax in tissue moulds and solidified on a cold plate. The blocks were then removed and sectioned with a rotary microtome (Leitz-Wetzlar) set at 5µm. The sections were mounted on glass slides, which were precoated with 'glue' (50% albumin and 50% glycerol) to prevent the loss of sections during staining. The slides were then dried overnight in an oven at 60°C before staining.

#### **b. Resin embedding**

A historesin embedding kit (LKB, Cambridge Instrument GmbH, D-6907, Germany) was used in this study. The following solutions were prepared prior to use.

### i. Infiltration solution

The Infiltration solution was made by dissolving 0.5 g of the Activator in 50 ml of Basic resin (with a magnetic stirrer). This solution could be kept in the dark at 4°C and used within several weeks.

### ii. Embedding medium

This medium was made up, by adding 1 ml of hardener to 15 ml of infiltration solution in a 50 ml glass beaker embedded in ice to slow down polymerization. The solution was stirred and mixed with a magnetic stirrer and used immediately.

### iii. Mounting medium

The mounting medium was made up and used immediately. Two parts of the powder component were mixed with one part of the liquid component. It was assumed that 1 g of the powder equalled 1 ml of the liquid.

Ovarian samples fixed with Bouin's solution and kept in 70% ethanol, were transferred to glass vials and dehydrated in a series of ethanol solutions through to 95% ethanol at 4°C. Due to the hydrophilic nature of the infiltration solution, complete tissue dehydration was not necessary. The samples were then slowly infiltrated manually through a series of



the infiltration solutions according to the procedure given in Appendix 1.2.2 at 4°C. When infiltration was completed, the samples were slightly translucent and sank to the bottom of the vials. At this point, the samples were ready to be embedded.

The samples were placed in an appropriate historesin mould tray and then filled with fresh embedding medium and allowed to polymerize overnight at room temperature. When the polymerization was completed, the excessive embedding medium was wiped off with absorbent tissue. Mounting medium was made up and poured onto the blocked polymerized samples. A 25mm section of a wooden broom handle was then placed on each block and allowed to solidify in the tray for 10-15 minutes. The blocks were then removed from the moulds and sectioned into a thickness of 5  $\mu$ m with a glass knife on a retracting microtome (Reichert-Jung 2050, Germany). The sections were then mounted directly onto precoated glass slides (as above) and dried overnight in an oven at 60°C prior to staining.

#### 2.2.1.2 Staining procedures

##### a. Haematoxylin and Eosin

The Haematoxylin and Eosin staining procedure given in Appendix 1.3.1 was used for general staining.

### **b. Heidenhain's Iron Haematoxylin**

Heidenhain's iron haematoxylin staining (Appendix 1.3.2) was used to observe both nuclear and cytoplasm components e.g., chromatin, nucleoli, yolk etc. In this study, it was used for detecting the distribution of chromosomes.

### **c. Polychrome Staining**

The polychrome staining technique has been used to detect the various stages of oocytes in the ovary of Atlantic mackerel (*Scomber scombrus* L.), yellow fin tuna, (*Thunnus albacares*) and Galapagos bacalao (*Mycteroperca olfax* Jenyns) (Coello, 1989) and also in *O. niloticus* (L) (Hamid, 1989). This technique enables the ovarian components such as the yolk granule, atretic oocytes and postovulatory follicles to be examined from a single slide. The staining procedure used in the present study (Appendix 1.3.3) was modified from Coello (1989) in order to obtain the best internal visualisation of *O. niloticus* oocytes.

### **2.2.2 Ultrastructural Studies**

Oocyte samples were dissected from the ovaries immediately after the fish were sacrificed. The samples were pre-fixed for 1 hour at 4°C in 2.5% glutaraldehyde (Taab Laboratories Equipment Ltd., Berkshine, England) in 0.1M phosphate buffer, and washed 3 times within 2 hours with 0.1 M

phosphate buffer. They were then post-fixed for 1 hour at room temperature with 1% osmium tetroxide (Taab Laboratories Equipment Ltd.) in 0.1M phosphate buffer, and washed 3 times within 2 hours with 0.1 M phosphate buffer. Next, the fixed samples were dehydrated in 50% ethanol for 1 hour, and then kept in 70% ethanol.

#### 2.2.2.1. Embedding medium

The embedding medium was made up shortly prior to use by emptying and thoroughly mixing a container of hardener and a bottle of accelerator into a container of Taab Embedding Resin Kit (Taab Laboratories Equipment Ltd.). After dehydrating the tissues through a series of ethanol concentrations, the fixed oocytes were further dehydrated in epoxy-propane (Taab Laboratories Equipment Ltd.) at room temperature and finally embedded according to the procedure given in Appendix 2.1.

Next, they were placed in gelatin capsules which contained a small drop of fresh embedding medium, and then filled up with the embedding medium. They were then incubated in a 60°C oven for 24 hours to complete polymerization. The samples were then sectioned.



#### 2.2.2.2. Microtomy

Embedded samples were sectioned into 60-90 nm thickness with an ultramicrotome (LKB Ultratome). The sections were fixed with chloroform and then mounted onto copper grids, which were dried on filter paper before staining.

#### 2.2.2.3. Staining

The grids containing the sections were stained with 20% uranyl acetate in absolute methanol by submerging them face up in a drop of uranyl acetate on a wax block for 2 minutes. The grids were then rinsed with fresh distilled water and blotted on a filter paper to remove excess water. The sections were stained with Reynold's lead citrate (Appendix 2.2) by placing the grid face down on a drop of lead citrate on a wax block for 2 minutes, and rinsed well with fresh distilled water. The stained grids were blotted on a filter paper before being examined and photographed using a transmission electron microscope (EM 300, Philip, Japan).

### 2.3 ESTIMATION OF OVARIAN VOLUME FRACTIONS ( $V_v$ )

The polychrome stained sections were examined under the microscope using the video camera linked to a monitor as shown in Figure 2.1. A multipurpose grid (M.168, Weibel,

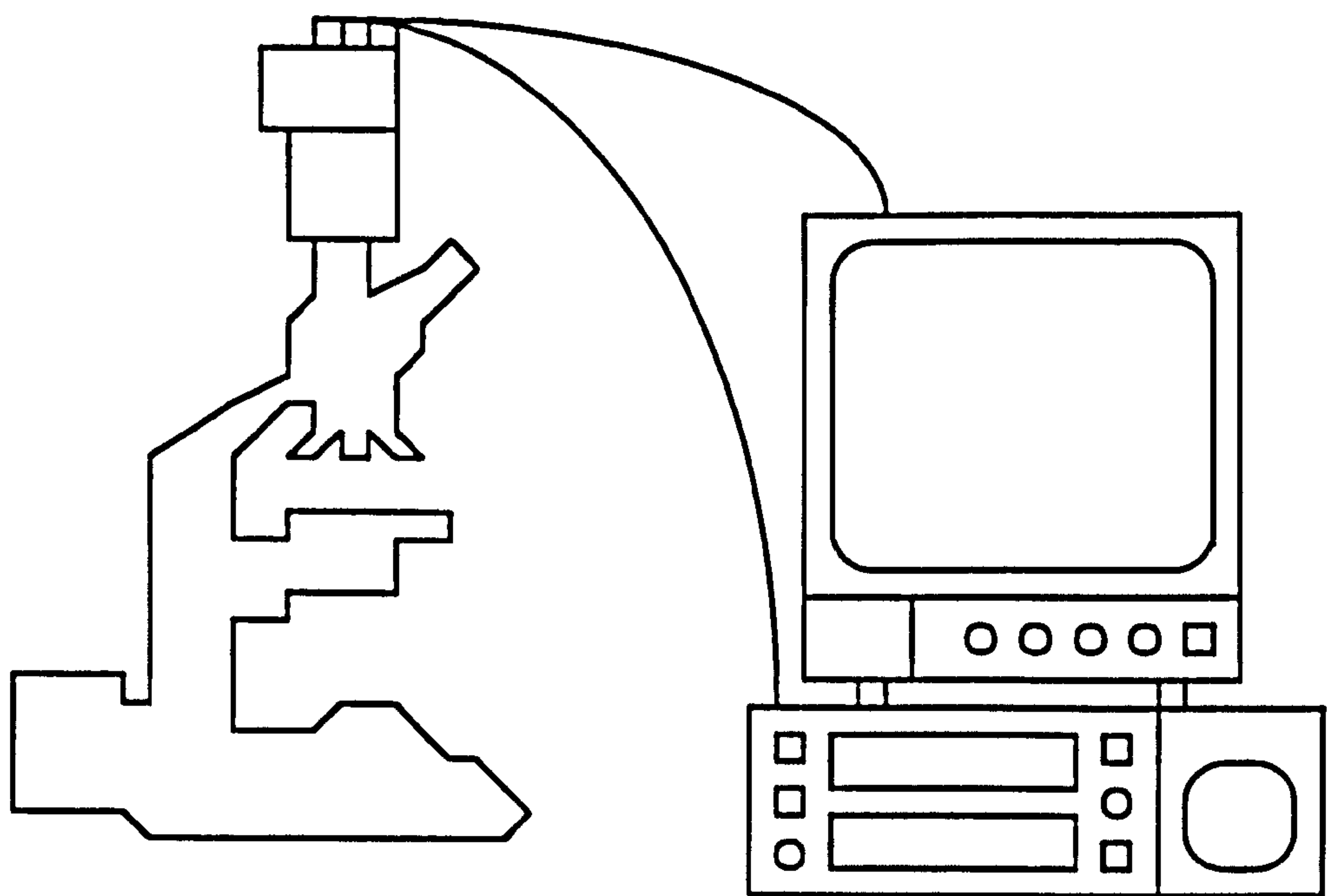
1979, Graticule Ltd, London) was placed in a photoeyepiece between the microscope and the video camera. The image of the grid was therefore overlaid on the ovarian section and viewed onto the monitor (Figure 2.1). Total number of grid points on the monitor and the numbers of the grid points lying within each oocytes stage were recorded and area fraction was computed from the equation given below, where  $A_{axp}$  = volume fraction ( $V_v$ ) of stage x oocytes.

$$A_{axp} = \frac{A_{sx}}{A_{ts}}$$

$A_{axp}$  = area fraction of oocyte stage x

$A_{sx}$  = area occupied with number of points in stage x

$A_{ts}$  = total point on the monitor



Microscope and Video camera

Video and monitor

Figure 2.1 Illustration of the microscope, video and monitor used to estimate the ovarian volume fractions ( $V_v$ ) of different oocyte stages (not to scale).



## 2.4 TOTAL CALCIUM ANALYSIS

Total calcium concentration in blood can be used as an index to estimate the levels of vitellogenin. (Elliott et al., 1984). In this study, the total calcium concentration in the serum or the plasma was analyzed with an atomic absorption spectrophotometer (AAS; Perkin-Elmer 2280, Basingstoke, Hants).

Samples were diluted 1:250-500 fold with the calcium diluting solution (Appendix 3.1) and kept overnight at 4°C prior to the further measurement. Calcium standards (BDH Ltd., Poole, England) were diluted with the calcium diluting solution to provide concentrations of 1, 2 and 4 mg/l. These standards were used for calibrating the spectrophotometer during measurement.

The Atomic absorption spectrophotometer was set up according to Appendix 3.3. The  $\text{Ca}^{2+}$  levels in the samples were then measured and recorded. Recalibration was carried out every 10-20 samples to ensure accuracy.

The concentration of calcium from the measurement, was multiplied by the dilution used and then divided by 10 to obtain the total calcium concentration (mg%) in each sample.

Pooled serum from males and females, which contained total calcium of 14 and 37 mg% respectively, were analyzed during each analysis for quality control throughout the study. The

intra-assay coefficient of variation was 5.3% and the inter-assay coefficient of variation was 9.2%.

## 2.5 STEROID RADIOIMMUNOASSAYS

The concentrations of testosterone and 17 $\beta$ -oestradiol in blood serum were determined according to the protocol established by Duston and Bromage (1987). The following solutions were made up before use.

- a. Phosphate buffer for the assay was made up according to procedure given in Appendix 3.2.
- b. The rabbit anti-testosterone antiserum (Steranti Research Ltd.) was raised against testosterone-3-(CMO)-BSA, prepared by o-carboxy-methyl-oxime formation at the steroid '3' position and coupling to bovine serum albumin(BSA). The rabbit anti-17 $\beta$ -oestradiol antiserum (Steranti Research Ltd) was raised against 17 $\beta$ -oestradiol-6-(CMO)-BSA prepared by o-carboxy-methyl-oxime formation at the steroid '6' position and coupling to BSA. The cross-reaction of both steroids is presented in Table 2.1. The freeze-dried antisera were dissolved in 1 ml of the phosphate buffer (Appendix 3.2) and 100  $\mu$ l aliquots transferred into new 10 polystyrene tubes which were then stored at -20°C until required. One tube of the 100  $\mu$ l aliquot was dissolved

and made up to 10 ml with phosphate buffer when required for an assay of 100 tubes.

- c. The radiolabels of [1,2,6,7-<sup>3</sup>H]testosterone (Amersham International Ltd., Bucks, U.K.) and [2,4,6,7-<sup>3</sup>H]oestradiol (Amersham International Ltd., Bucks, U.K.) were obtained in 250  $\mu$ Ci quantities 'stock label'. The working label solution of testosterone or 17 $\beta$ -oestradiol was made up by diluting 10  $\mu$ l of the stock label with 2 ml absolute ethanol. An aliquot of the intermediate solution was dried down under nitrogen gas and reconstituted with the phosphate buffer to prepare a working solution of approximately 20,000 dpm/100  $\mu$ l.
- d. Stock standard solutions of testosterone or 17 $\beta$ -oestradiol were prepared by dissolving 1  $\mu$ g testosterone or 17 $\beta$ -oestradiol in 10 ml of absolute ethanol (100 ng/ml). These stock standards were stored at -20°C. Prior to starting each assay, 100  $\mu$ l of the stock standard was thawed and diluted with 1 ml absolute ethanol (10 ng/ml) 'working standard'. The standards were then prepared by diluting the working standard with absolute ethanol to obtain a series of standards containing 7.8, 15.6, 31.3, 62.5, 125, 250 500 and 1000 pg/tube.



Table 2.1: Cross-reaction of testosterone and 17 $\beta$ -oestradiol with similar steroids

| Steroids   | Cross-reaction (%)     |                                  |
|--|------------------------|----------------------------------|
|  | Testosterone antiserum | 17 $\beta$ -oestradiol antiserum |
| 17 $\beta$ -oestradiol                               | 5.8                    | taken as 100                     |
| Testosterone   | taken as 100           | 1.6                              |
| Oestrone   | 2.9                    | 7.5                              |
| Oestriol   | 1.0                    | 12.2                             |
| 11-Ketotestosterone                                  | 34.5                   | 1.0                              |
| Androstenedione                                      | 1.0                    | 1.8                              |
| 17 $\alpha$ -hydroxyprogesterone                     | 1.0                    | 1.0                              |
| 17 $\alpha$ -hydroxy-20 $\beta$ -dihydroprogesterone | 1.66                   | 1.0                              |
| Pregnenolone   | 3.3                    | 1.0                              |
| Cortisol   | 1.0                    | 1.0                              |

From Duston and Bromage (1987)

- e. The dextran-coated charcoal solution was made up by dissolving one tablet of the Separex dextran-coated charcoal tablet (Steranti Ltd., U.K.) into 50 ml phosphate buffer (Appendix 3.2) and stirred continuously on ice for 30 minutes prior to use.

### 2.5.1 Extraction of Steroids for Assays

The steroid in samples were extracted by adding 100  $\mu$ l of the serum and 2 ml of ethyl acetate into polypropylene tubes (Lt4, Luckhams Ltd.). The tubes were then attached on to a rotary mixer and mixed for 1 hour. Next, the sample tubes were centrifuged at 1,500 rpm (4°C) for 10 minutes. The extracted samples were stored at 4°C until required.

### 2.5.2 Assay Procedures

Fifty  $\mu$ l (for testosterone analysis) or 100  $\mu$ l (for 17 $\beta$ -oestradiol analysis) of the extracted samples were transferred into rimless soda glass tubes (R.B.Radley and Company Ltd., Herts, U.K.) prior to the assays. A series of standard dilutions were prepared with absolute ethanol in the rimless soda glass tubes to cover the range of 0-1000 pg/100  $\mu$ l. The extracted samples and the standards were then vacuum dried in an oven maintained at 20 to 30°C. The dried tubes were then cooled to 4°C.

One hundred  $\mu$ l of anti-testosterone or anti-17 $\beta$ -oestradiol and 100  $\mu$ l of testosterone or 17 $\beta$ -oestradiol labels were added into the cooled dried tubes. All the tubes were thoroughly mixed for 10 seconds and incubated overnight at 4°C.

Next, 0.5 ml of dextran-coated charcoal solution (as above) was added into each tube and well mixed. The sample and standard tubes were incubated at 4°C for 10 minutes. They were then centrifuged at 2000 rpm (4°C) for another 10 minutes and 0.4 ml of the supernatant from each tube was transferred into a scintillation vial containing 4 to 9 ml of scintillation fluid (Gold; Canberra Packard).

The vials were mixed thoroughly and the radioactivity counted for 5 minutes in a scintillation counter (Tri-carb 2660/2000CA; Canberra Packard). Each assay included 3 extra vials. The first two vials of these contained 100 µl of the tritiated hormone and scintillation fluid for estimating the total radioactivity and the third vial contained only scintillation fluid as a blank for automatic subtraction of the background counting.

### 2.5.3 Calculation of Steroid Concentration

Means of total dpm were multiplied by 0.54 for correcting the difference between the total solution volume per tube (0.7) and the volume of the supernatant taken (0.4). Percentage binding was computed as follows:

$$\text{Percentage binding} = \frac{\text{dpm of standard or samples}}{\text{mean of total dpm}} \times 100$$



The percentage binding was plotted against the concentration on log-linear graph paper. The concentrations of the samples were then read from the standard curve.

The concentrations were multiplied by 0.42 (for testosterone) or 0.21 (for 17 $\beta$ -oestradiol) to correct for the sample volume extraction (50 or 100  $\mu$ l from 2.1 ml), the volume of extracted serum used (100  $\mu$ l) and then converted to ng/ml (1/1000).

Sensitivity of the assays was defined as the quantity of testosterone or 17 $\beta$ -oestradiol, statistically distinguishable from the zero standard. This lower limit was 7.8 pg/tube.

#### 2.5.4 Quality Control

The pooled serum of males and females, which contained 64.63, 52.45 ng/ml testosterone and 5.5 and 13.7 ng/ml 17 $\beta$ -oestradiol, respectively, were analyzed throughout the study for inter and intra-assay quality control. The intra-assay and the inter-assay coefficient of variations for testosterone were 3.8 and 12.8%, respectively. Similarly, the intra-assay and inter-assay coefficient of variations for 17 $\beta$ -oestradiol were 6.0 and 16.8%, respectively.

## 2.6 Statistical Methods

Unless otherwise stated, data were presented as mean $\pm$ SE. Statistical methods were analyzed according to Sokal and Rohlf (1981) using Minitab statistical package (Ryan, Joiner and Ryan, 1981) which was performed on a Hewlett-Packard mainframe and personal computer. For means with homogenous variances, either one-way or two-way analysis of variance (ANOVA) was used to compare two or more sample means. When the sample variances were heterogenous, non-parametric analysis (Mann Whitney) was used to detect differences between groups. The significance was noted at the 5% level.

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## CHAPTER 3

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### 3. CLASSIFICATION OF OOCYTE DEVELOPMENT INTO DIFFERENT STAGES OF *Oreochromis niloticus*.

#### 3.1 INTRODUCTION

There are several reports and reviews on the macroscopic and microscopic structures of gonads in various teleost species (Dadzie, 1970; Takahashi, 1974; Macer, 1974; Babiker and Ibrahim, 1979a; Wallace and Selman, 1981; Nakahama, 1983; Guraya, 1986; Wallace, Selman, Greeley, Begovac, Lin and McPherson, 1987; Hoffmann and Meder, 1988; Bromage and Cumaranatunga, 1988; Selman and Wallace, 1989). These publications, however, often use different criteria to categorize the various stages of gametogenesis.

In general, gametogenesis can be divided into distinct phases based on the morphology, biochemistry and molecular transformation of the gonadal cells. In teleosts, the development and differentiation of oocytes involves: 1) formation of the chorion, 2) formation of numerous nucleoli and development of lambrush chromosomes, and 3) accumulation of various RNA and variety of yolks in the ooplasm.

### 3.1.1 Gonadal Development in Teleosts

#### 3.1.1.1 Sexual differentiation of gonads

Sexual differentiation in poikilothermic animals has been widely studied. The onset of differentiation depends on the interaction between sex (Nakamura and Takahashi, 1973) and temperature (Bull and Vogt, 1979; Conover and Kynard, 1981). The meiotic prophase division (leptotene) in the germ cells, is the most widely used criterion for determining sexual differentiation. Using this criterion, Nakamura and Takahashi (1973) reported that the onset of sexual differentiation occurred earlier in *O. mossambicus* females than in males. In addition to the morphology of the germ cells, developments such as the formation of ovarian cavity or testicular lumen, can be used to detect early sexual differentiation of some species. In *O. mossambicus* for example, formation of the ovarian cavity and meiotic germ cells occurred at about 20 days after hatching at 20°C (Nakamura and Takahashi, 1973). Meanwhile for the testicular development of the same species and temperature, the first appearance of the efferent duct was observed in the same period as the formation of the ovarian cavity but the meiotic division of the germ cells did not occur until 50 - 60 days post-hatch (Nakamura and Takahashi, 1973). Whereas Alvendia-Casual and Carino (1988) reported the onset of sex differentiation in *O. niloticus* fry at 30 - 33 days post-hatching (25 - 26°C).

### 3.1.1.2 Localization and morphology of ovarian structures

In teleosts, the ovaries are paired elongated structures situated in the postero-dorsal aspect of the body cavity and ventral to the swim bladder (Sathyanesan, 1961).

Wallace and Selman (1981) and de Vlaming, Grossman and Chapman (1982) have classified mature ovaries into three basic groups or types according to the pattern of oocyte development. The first group is the "synchronous ovary", which contains oocytes at the same stage of growth and all of the oocytes ovulate from the ovary in unison. This ovarian pattern is usually found in teleosts, which spawn only once in their life and then die (semelparous) (e.g., anadromous, *Oncorhynchus* species or the catadromous eel). The second type is the "group-synchronous ovary", where at least two populations (groups) of larger and smaller oocytes can be distinguished in the same ovary at the same time (iteroparous). In spawning fish, these larger oocytes are ovulated while the small oocytes remain in the ovary. The smaller oocytes which are in endogenous vitellogenic stage, commence development to the larger oocytes and then ovulate in the subsequent breeding. In some cases of the oocyte recruitment, the smaller oocytes develop from endogenous to exogenous vitellogenesis and may rapidly progress to maturation, or may be temporarily arrested in late vitellogenesis prior to further maturation. This second pattern is commonly found in teleosts which spawn



once a year (e.g. flounder, *Liopsetta abscura* and rainbow trout, *Onchorynchus mykiss*). The third type is the "asynchronous ovary". Oocytes at different developmental stages can be found without a dominant oocyte population. The process of oocyte recruitment, occurs among all oocyte stages as a continuous process during the breeding season. This pattern is found in the killifish, *Fundulus heteroclitus* and syngnathoids (e.g. pipefish and seahorse).

#### 3.1.1.3 Oogenesis in teleosts

Oogenesis is a process whereby immature oocytes develop into mature oocytes or ova. Oogenesis can be divided into various stages depending on the morphology of the nucleus, cytoplasm and follicle (Dadzie, 1970; Selman and Wallace, 1983; 1989; Nakahama, 1983; Cumaranatunga, 1985 and Wallace et al., 1987). These phases have been preliminary grouped into previtellogenic, vitellogenic and maturation.

##### a. Primary growth (previtellogenic oocytes)

###### Stage 1: Chromatin nucleolar stage

Chromatin nucleolar stage, is characterized by a conspicuous nucleolus associated with the chromatin threads. This stage develops from oogonia, which have undergone mitotic proliferation, and then enters the early stages of the first meiotic division. During the first

meiotic stage (prophase), the DNA begins to replicate (leptotene), the homologous chromosomes pair up and condense (zygotene), the chromosomes shorten and thicken to form synaptonemal complexes (pachytene) and finally the chromosomes take on a "lampbrush" configuration (diplotene). At this stage, the nucleus of the oocytes contains only one nucleolus and the oocytes are arrested. These oocytes are called the primary oocytes (Dadzie and Hyder, 1976; Nagahama, 1983).

### **Stage 2: Perinucleolar stage**

Following a short quiescent period in meiotic prophase, the oocytes enter a growth phase; increasing in size progressively and becoming surrounded by a layer of granulosa cells. The ribosomal genes, in the nucleus of the oocytes at this stage, begin to amplify and the nucleus multiply into several nucleoli (Anderson and Smith, 1978). The nucleoli generally lie in the peripheral region of the enlarging oocyte nucleus (germinal vesicle). In addition, RNA and mRNA are transported from the germinal vesicle (nucleus) into the oocyte cytoplasm, and appear as an aggregation of basophilic material. These materials have been termed as "yolk nuclei" or "Balbiani bodies". The Balbiani bodies are composed of RNAs and cytoplasmic organelles e.g., mitochondria, multi-vesicular bodies, endoplasmic reticulum and Golgi element (Guraya, 1986; Selman and Wallace, 1989). The Balbiani bodies initially

form at the juxtannuclear position and subsequently migrate to the oocyte periphery and then disperse into small fractions (Guraya, 1986). At the end of stage 2, the oocytes are surrounded by a multi-follicular layer consisting of rested granulosa layer, a vascularized thecal layer and a thin surface epithelium.

The Balbiani body is well documented in many species (see Beams and Kessel, 1973; Guraya, 1986) but its function is poorly understood. Mostly, it has been suggested to be involved in the extensive fabrication of organelles in the growth of oocytes (Guraya, 1986; Selman and Wallace, 1981, 1989; Wallace et al., 1987).

During the growth phase of stage 2 oocytes, their volume increases approximately a thousand-fold from a diameter ranging from 10 - 20  $\mu\text{m}$  (leptotene) to 100 - 200  $\mu\text{m}$  (stage 2). This increase in oocyte volume is due to the elaboration of normal cytoplasmic organelles and the accumulation of cytoplasmic RNAs and proteins. The ovaries of fish at this stage contain immature oocytes and have a gonadosomatic index of less than 2% (Wallace and Selman, 1981; Wallace et al., 1987 and Selman and Wallace, 1989).



## **b. Secondary growth (vitellogenic oocytes)**

During this growth phase the oocytes increase in size and result from two forms of growth: endogenous and exogenous vitellogenesis. Initial oocyte size increases are due to endogenous vitellogenesis. This is then followed by the incorporation of vitellogenic products from liver. These two phases are accompanied by numerous internal structure changes.

### **Stage 3, Stage 4: endogenous vitellogenic stage**

At the onset of endogenous vitellogenesis, the Balbiani bodies completely disappear from the oocyte cytoplasm, (stage 3). At the end of the endogenous vitellogenesis (stage 4), the cortical alveoli are presented throughout the oocyte cytoplasm (Wallace et al., 1987; Selman and Wallace, 1989). In fact, the cortical alveoli have been given several terms: cortical vesicles, yolk vesicles, intra vesicular yolk and endogenous yolk; but they should not be referred to as yolk because they do not contain yolk nutrient sources for embryo (Selman and Wallace, 1981; Selman, Wallace and Barr, 1986; Wallace et al., 1987; Selman and Wallace, 1989). Therefore, the term "cortical alveoli" has been used throughout the present study.

Autoradiographical studies in zebrafish, *Brachydanio rerio* (Korfmeier, 1966) demonstrated and concluded that the composition of cortical alveoli was a glycoprotein, which was synthesized in the oocyte. The formation of cortical alveoli has been examined in developing oocytes of *Fundulus heteroclitus* (Selman and Wallace, 1983; Selman et al., 1986; Wallace, 1985), from which it was concluded that, the cortical alveoli contained an endogenous carbohydrate product. In addition, the results from ultrastructure studies implicated the Golgi and/or endoplasmic reticulum in stage 4 development. A >200-kDa glycoconjugated protein was detected in the cortical alveoli using immunocytochemical techniques (Selman, Wallace and Barr, 1986). Thus, the cortical alveoli are usually stained for protein and carbohydrate with Alcian blue, toluidine blue and periodic and Schiff reagents (Selman et al., 1986). Cortical alveoli, however, are difficult to preserve during tissue preparation with normal oocyte fixation. Therefore, they frequently lose their staining characters and assume a vacuolar appearance (Wallace et al., 1987).

At the end of stage 4, cortical alveoli continue to form and be displaced to the periphery of the oocytes. The oocyte diameters at the end of endogenous vitellogenesis increase up to 0.6 - 0.9 mm in *S. niloticus* (Jalabert and Zohar, 1982).



### **Stage 5: exogenous vitellogenic stage**

In this stage, the oocytes enter a prolonged growth phase, which is under the control of pituitary gonadotropins. In lower vertebrates including the teleosts, enlargement of oocytes is mainly due to the accumulation of yolk. Many studies on amphibians and birds indicate a sequence of extra gonadal events that exclusively contributes to vitellogenesis. These events include an increase in hepatic synthesis and secretion of vitellogenin, lipophosphoprotein and yolk protein precursor in response to circulating oestrogen. The vitellogenin is delivered via the maternal bloodstream to the surface of the growing oocyte where it is selectively transported by receptor-mediated endocytosis into the oocytes. Within the oocytes, vitellogenin forms yolk bodies by micropinocytosis. The vitellogenin finally changes into polypeptide sub-units of yolk proteins; lipovitellin and phosvitin (Wallace, 1985).

In many teleosts, these yolk proteins; which contributes to over 80 - 90% of the egg dry weight (Wallace et al., 1987); uniquely accumulates in fluid-filled yolk spheres of yolk globules. The yolk spheres may either maintain their integrity throughout oocyte growth or fuse centripetally to form a continuous mass of fluid yolk (Wallace and Selman, 1981) giving many teleost eggs a transparent like character (Wallace and Selman, 1981; Wallace et al., 1987; Selman and Wallace, 1989).



### **c. Maturation**

**Stage 6: germinal vesicle migration stage**

**Stage 7: ovulation stage**

When an appropriate hormone (e.g. pituitary gonadotropins) stimulates oocytes at the end of vitellogenesis, the oocytes eventually undergo maturation (stage 6) and ovulation (stage 7) (Masui and Clarke, 1979). In oocyte maturation; stage 6, oocytes are characterized by the migration of the germinal vesicle (GVM) towards the animal pole of the oocyte cytoplasm. This is accompanied by the concomitant dissociation of the nuclear envelope. Subsequently, when germinal vesicle break down (GVBD; stage 7) occurs, the oocytes are hydrated and released (ovulation) into the ovarian cavity. At this time, the chromosomes of the oocytes condense and complete the first meiotic division. These oocytes (ova) are fertilisable and the fertilisation of the ovum stimulates the second meiotic division which is followed by combination of the male and female chromosomes. At this stage, the oocytes have reached their final size and maturation. The maturation of oocytes in teleosts is usually rapid and accomplished within 24 hours. (Wallace and Selman, 1981; Wallace et al., 1987; Selman and Wallace, 1989).

In marine teleosts, pelagic eggs are buoyant. An enormous oocyte volume increase is due to rapid water uptake (Fulton, 1898; Hirose and Ishida, 1974; Hirose, Machida and Donaldson, 1976), and concomitant increase in  $K^+$  and  $Cl^-$  (Watanabe and Kuo, 1986). For example *in vivo* studies in grey mullet, *Mugil cephalus*, had shown that when the fish were injected with partially purified salmon gonadotropin (SG-G100), ovulation and oviposition occurred after 8 and 12 hours post injection, respectively. The water content of these oocytes at oviposition increased to the maximum of 84.8% compared to 59.4% in the oocytes prior to the ovulation (Watanabe and Kuo, 1986).

During oocyte development, all the above physiological events do not sequentially replace one another but rather the events are sequentially initiated and remain active throughout the oocyte development. Oocyte growth and the processes previously initiated, may be accelerated or slowed down by the influence of a regime of hormones and/or can even cease during atresia (Wallace and Selman, 1981; Wallace et al., 1987; Selman and Wallace, 1989).

#### d. Atresia

Atresia is a degenerative process during which oocytes in various stages stop their development and undergo rupture or resorption *in situ*. These oocytes have been referred to by many names e.g., aborted egg, corpora lutea atretica, preovulation corpus luteum, hyaline oocyte, post-ovulatory degeneration of egg but mostly as atretic oocytes (Sathyanesan, 1961; Macer, 1974; Robb, 1982; Babiker and Ibrahim, 1979a; Hastings, 1981; Nakahama, 1983; Guraya, 1986; Abu-Hakima, 1987).

Various factors have been implicated in follicular atresia including age of the fish, stage of reproductive cycle, hormones of extraovarian and intraovarian sources, captivity, light, temperature, crowding, nutrition, ischaemia, irradiation, etc (Guraya, 1973; 1986).

Follicular atresia in teleost ovaries occurs commonly during pre-spawning, spawning and post-spawning periods. Mostly the vitellogenic oocytes and unshed ova are affected (Nakahama, 1983; Guraya, 1986). In fact, there is no difference between the sizes of developing and atretic oocytes. Thus, the fecundity estimates based on oocyte size can be affected by atresia (Macer, 1974). In rainbow trout, (*Onchorynchus mykiss*) for example, 10 to 30% of the vitellogenic oocytes were found to be atretic oocytes (Bromage and Cumuranatunga, 1988).



There are many changes occurring during the process of oocyte atresia. These processes were reviewed by Cumaranatunga (1985), and were divided into  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\sigma$ . During the first stage of oocyte degeneration ( $\alpha$ ), the oocytes lose their water and shrink. At the same time the oolemma and oocyte contents are resorbed as a result of phagocytosis by the granulosa cells. The yolk liquifies, forming irregular drops and its acidophilic nature diminishes producing basophilic granules. Then, the nucleus ruptures and releases its contents into the ooplasm. The  $\beta$  stage is characterized by an invasion and multiplication of follicle cells; at this stage, numerous blood vessels have been observed in the ovary. The  $\gamma$  stage is the stage that completes follicular disintegration. During this stage, the presence of 17- $\beta$  hydroxy-steroid dehydrogenase (17 $\beta$ -HSD) had been reported (Khoo, 1975). The  $\sigma$  stage, which is the final stage of the atretic process, is characterized by the presentation of "brown bodies". These are the residual bodies; brown or black in colour, within the regressing follicles. Khoo (1975) described the fifth atretic stage as the  $\Sigma$  stage and suggested that this stage involved oogenesis, because many oogonia were present in post-ovulated ovaries.

#### 3.1.1.4 Oogenesis in Cichlids

There are several studies on the gametogenesis of wild cichlid stocks originating from open waters. These studies were based on fish, whose age structure and spawning history are often unknown. For example, macroscopic and microscopic structures of oocytes have been studied in *O. niloticus* from the White Nile (Babiker and Ibrahim, 1979a), Lake Nasser (Latif and Saady, 1973b), Lake Manzalah (Hussein, 1984). The morphology of gonadal development in *O. mossambicus* had also been studied in captivity (Dadzie, 1970), *O. zillii* (Dadzie and Wangila, 1980) and *O. niloticus* (Lin Yun, Liu Guoan, Chen Shuqun and Lui Chuwu, 1983).

Unfortunately, all these reports classify oocyte development into superficial phases. For example, Jalabert and Zohar (1983) divided the oocyte growth of tilapias into previtellogenic and vitellogenic growth phases. In addition, other studies described the oocyte development as immature, mature, ripe, ripe running and spent oocytes (Dadzie, 1970; Latif and Saad, 1973b; Babiker and Ibrahim, 1979a; Dadzie and Wangila, 1980; Hussein, 1984). Moreover, atretic oocytes have been excluded from all these oocyte classification studies. Thus, oocyte development is based on a population of unknown reproductive history and the oocyte classification of tilapias to date is incomplete.

### 3.2 OBJECTIVES

In the present study, *O. niloticus* broodstocks of known age and spawning history were reared under regulated hatchery conditions. The female gonad was used to determine sexual differentiation and classify oocyte development. The classification of oocyte stages were then used throughout the study.

### 3.3 METHODS

The experimental fish used in this investigation were obtained from the genetically pure *O. niloticus* stock held at the tropical aquarium, Stirling University. They were maintained at the same temperature and photoregime as described in section 2.1. Fish were randomly sampled twice a week after hatching for 24 weeks. The weight (g) and total length (cm) of the fish were recorded.

The gonads selected for this study were dissected and then fixed in Bouin's solution. Immature gonads were processed with paraffin wax while mature female gonads were processed with resin (section 2.2.1). The samples were sectioned at the thickness of 4 - 5 $\mu$ m and stained with either haematoxylin and eosin, Heidenhain's haematoxylin or polychrome (section 2.2.1.2) and examined under a



microscope. Representative oocyte stages were photographed (Olympus, BH2). Gonadal stages were identified according to criteria of Dadzie (1970), Selman and Wallace (1981, 1989), Cumaranatunga (1985) and Wallace et al. (1987).

### 3.4 RESULTS

Oocyte stages were classified on the basis of their nuclear structure, cell sizes, transformation of the cells, different inclusions in cytoplasm and the presence of some structures and cells of the follicle i.e., granulosa, theca, zona pellucida. The development of oocytes after sexual differentiation, was divided into primary growth (two stages), secondary growth (three stages), maturation (two stages) and atresia (one stage).

#### 3.4.1 Gonadal Sexual Differentiation

##### 3.4.1.1. Primordial germ cells

Primordial germ cells (PGCs) were observed in newly hatched fry, which were 5.6 - 5.7mm in total length. The PGCs were located in pairs along the dorso-median part of the peritoneal wall at the dorsal root of the mesentery (Plate 3.1a). The PGCs were oval shaped and easily distinguishable

from other somatic cells by their clear cytoplasm and large nucleus. The diameters of PGCs and nuclei were  $12.7 \pm 0.59\mu\text{m}$  and  $7.5 \pm 0.48\mu\text{m}$ , respectively.

#### 3.4.1.2. Undifferentiated gonadal-sacs

Undifferentiated gonadal-sacs were first observed in 3 day old post-hatched fry measuring 6.0 - 7.5 mm in total length. The gonadal-sacs were suspended from the dorsal peritoneal wall into the coelomic cavity by a thin sheet of mesogonium (Plate 3.1b). These sacs contained a small number of mitotic cells (oogonia). The oogonia which were irregular in shape, contained very little cytoplasm and increased in numbers rapidly by 7 days post-hatching (8.5-11mm). Active mitotic cells were also observed in 11 day old post-hatched fry (8.9 - 13.4 mm).

#### 3.4.1.3. Sexually differentiated gonads

Sexually differentiated gonads were observed in 14 day old post-hatched fry (11.5 - 15.4mm). At this period, some of the germ cells in the gonads began their development into meiotic prophase. The presence of a ovarian cavity and efferent ducts were also observed during this period (Plate 3.2).

### **3.4.2 Ovarian Development and Oogenesis**

#### **3.4.2.1. Primary growth (previtellogenic oocytes)**

##### **a. Oogonia**

At this stage, the oogonia were commonly found in small nests in the germinal tissue. The oogonia proliferated by mitotic division for the subsequent breeding cycle. These cells were irregularly shaped, contained very little cytoplasm and ranged from 2 to 5  $\mu\text{m}$  in diameter (Plate 3.2; 3.3a).

##### **b. Stage 1 oocytes (chromatin nucleolus stage)**

During stage 1, the oogonia began meiotic prophase (leptotene). The chromosomes in the nucleus became conspicuous, as the homologous chromosomes paired (zygotene), shortened and thickened (pachytene), and then underwent chromosomal configuration (diplotene) (Plate 3.3a). The size of the cells increased from 5 to 52  $\mu\text{m}$  in diameter. The nucleus also increased in size and contained only one nucleolus and several strands of basophilic chromatins (Plate 3.3b; 3.4a). The cytoplasm was composed of an indistinct narrow band around the nucleus. The stage 1 oocytes were initially observed in 21 day old post-hatched fry (Plate 3.3b).



### c. Perinucleolus stage (stage 2 oocytes)

Stage 2 oocytes were subdivided into three phases according to the position of their Balbiani bodies. In the early stage 2 oocytes [2A], the Balbiani bodies were located close to nuclear envelop and stained strongly basophilic (dark purple-black) with polychrome stain (Plate 3.5b; 3.6a,b; 3.7a,b). In mid stage 2 oocytes [2b], the Balbiani bodies were distributed all over the oocyte cytoplasm and stained dark basophilic (purple) with polychrome (Plate 3.5b; 3.6a,b; 3.7a,b). Finally, in the late stage 2 oocytes [2c], Balbiani bodies (light grey) migrated to the periphery of the oocyte cytoplasm (Arrows) (Plate 3.5; 3.6; 3.7b).

The diameter of stage 2 oocytes ranged from 50 to 205  $\mu\text{m}$ . The nucleus of the cells also increased in size and contained several nucleoli which were distributed around the periphery of the nucleus. Chromatins were easily detected as basophilic strands distributed throughout the nucleus (Plate 3.6a,b).

As the cytoplasm increased in volume, the basophilic cytoplasm (purple-black) was easily detected by the polychrome staining technique (Plate 3.6a,b). The basophilic nature of oocyte cytoplasm had gradually reduced in concomitant with the oocyte development. The follicle of oocytes at stage 2c was distinguished as two thin layers

comprising of a granulosa and a thecal layer (Plate 3.6b). In *O. niloticus*, the stage 2 oocytes were initially detected in 28 day old post-hatched fry (Plate 3.4a) and had appeared commonly in every ovarian samples (Plate 3.4b; 3.5; 3.6; 3.7).

#### 3.4.2.2 Secondary growth oocytes (vitellogenic oocytes)

##### a. Endogenous vitellogenesis

##### Stage 3 oocytes

In this stage, the oocytes ranged from 110 to 340  $\mu\text{m}$  in diameter. The nucleus contained several nucleoli which were located close to the undulated nuclear envelope (Plate 3.5a; 3.7a,b) and the Balbiani bodies had completely disappeared from the cytoplasm. Thus, the cytoplasm of the oocytes was stained light grey or light blue with polychrome (Plate 3.7a,b). Numerous vacuoles appeared close to the nucleus and moved to the periphery of the cytoplasm. These vacuoles stained as PAS positive, due to glycoprotein composition of the vacuoles (Plate 3.11a). The developing follicle of the oocytes in this stage, consisted of a cuboidal granulosa layer and thecal layers (Plate 3.10b; 3.11a).

#### **Stage 4 oocytes**

The diameter of stage 4 oocytes ranged from 172 to 683  $\mu\text{m}$ . The oocytes had rapidly increased in size as a result of the accumulation of endogenous carbohydrate and other trophoplasmic substances. The nucleus contained several nucleoli which were located in the highly undulated nuclear envelope (Plate 3.7a,b). The cytoplasm occupied a great proportion of the oocyte and stained acidophilia i.e., light pink (Plate 3.7a,b; 3.11a) with polychrome. In addition, the developing chorion or zona pellucida became more conspicuous in this stage (Plate 3.10a; 3.11a).

#### **b. Exogenous vitellogenesis**

##### **Stage 5 oocyte**

The oocytes which were classified as stage 5, ranged from 214 to 970  $\mu\text{m}$  in diameter. These oocytes were easily distinguished by the presence of yolk granules in the ooplasm and stained mauve blue with polychrome (Plate 3.8a,b). These granules, which subsequently accumulated into yolk pellets (yolk globule), were distributed throughout the cytoplasm. The yolk globules increased in size, due to the fusion of smaller yolk granules and stained a strong mauve blue in late stage 5 (Plate 3.8b). In addition, there were numerous vacuoles around the



periphery of the cytoplasm. The nucleus contained a few nucleoli and were generally located at the centre of the oocytes (Plate 3.8a,b).

### c. Maturation

#### Stage 6 oocytes

The oocytes in this stage ranged from 422 to 1,965  $\mu\text{m}$  in diameter. The nucleus migrated towards the animal pole of the oocyte cytoplasm and was reduced in size; it contained indistinct nucleoli and was surrounded by a highly folded nuclear envelope (Plate 3.9). There were many vacuoles (cortical alveoli or oil droplets) located close to the follicular layers; the layers were thicker than those of stage 5, due to an increase in the sizes of the granulosa and the thecal cells (Plate 3.10a,b; 3.11a,b). Some of the yolk granules were fused to form yolk platelets. The size of the platelets at the animal pole was smaller than those at the vegetal pole. The zona pellucida was also well developed and clearly separated into two layers; the zona interna and zona externa (Plate 3.10a,b).

#### d. Atresia

Three atretic stages were commonly found in stage 5 and 6 oocytes in pre-spawned and post-spawned *O. niloticus* ovaries. In the first stage of atresia ( $\alpha$ ), the nucleus had collapsed and released its contents into the cytoplasm (Plate 3.12a). Subsequently, the oocytes had begun to shrink and separate from the hypertrophied granulosa layer ( $\alpha$  stage; Plate 3.12b). The affinity of yolk in these oocytes, had changed from basidophilia into acidophilia. These acidophilic regions began at the periphery of the cytoplasm (Plate 3.12a; 3.13a,b). In advanced atresia ( $\beta$  and  $\gamma$ ), disorganized and acidophilic liquefied yolk was observed. The remaining oocyte mass was invaded by the infolding of the follicular layers and was ultimately resorbed (Plate 3.13a). At the end of this  $\gamma$  stage, the remainder of the liquid yolk fraction could still be detected in acidophilic yolk and the follicles had completed disintegration (Plate 3.13b).



Plate 3.1 Transverse section of a newly hatched *O. niloticus* fry (day 0) showing:

(3.1a) Primordial germ cells. Section of gonads stained with haematoxylin and eosin. x1,000

(3.1b) Undifferentiated gonadal sac of 5 days post-hatched fry. Sections stained with haematoxylin and eosin. x1,500

PGCs primordial germ cells

MS mesonephric duct

GUT gut

GC germ cells

ME mesogonium



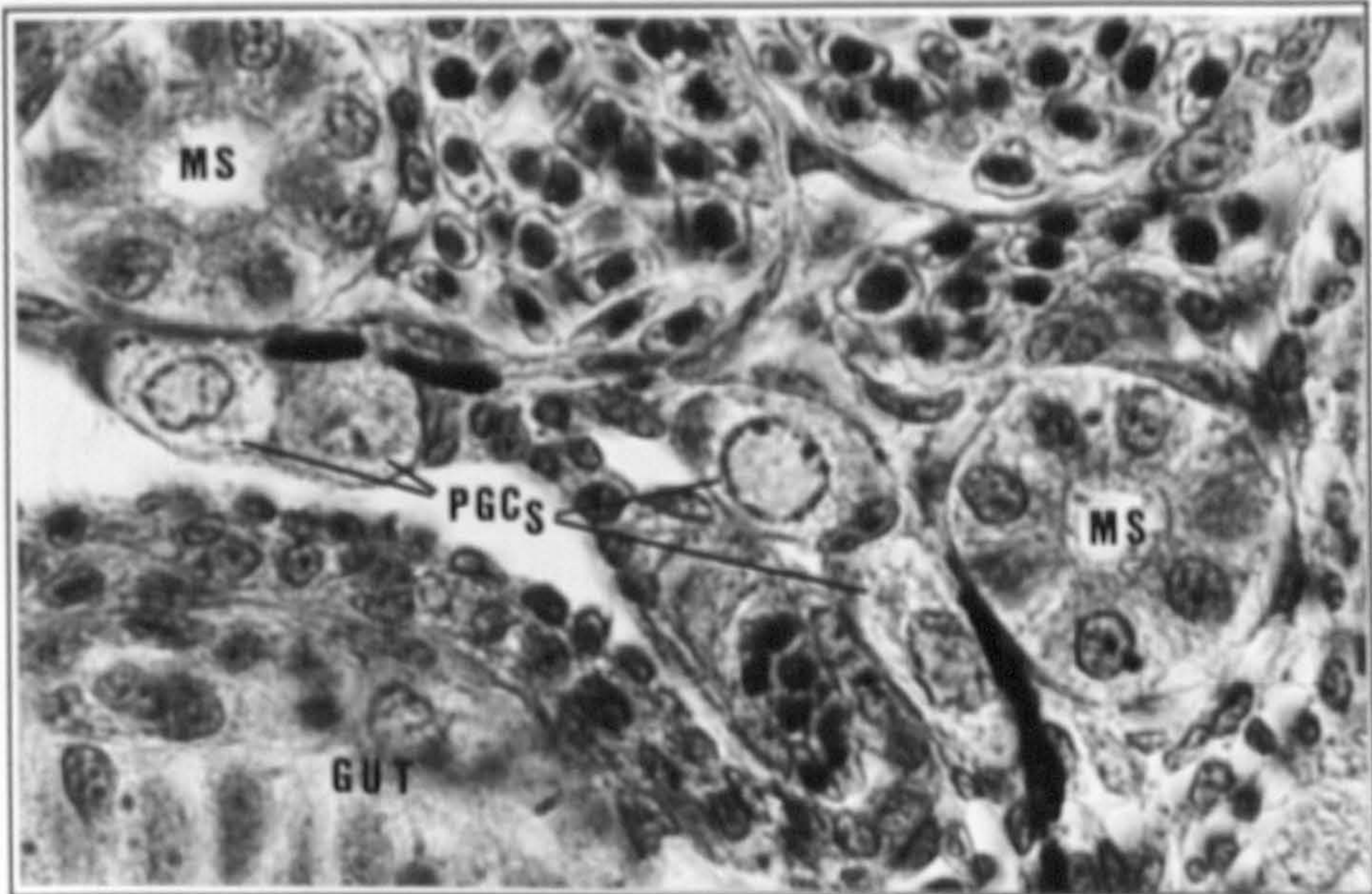


Plate 3.1a



Plate 3.1b



Plate 3.2 Transverse sections of female sexually differentiated gonads of 14 days post-hatched *O. niloticus* fry ( $27\pm 1^{\circ}\text{C}$ ). Section stained Heidenhain's haematoxylin. x1,000

|     |                    |
|-----|--------------------|
| ME  | mesogonium         |
| OG  | oogonia            |
| LEP | prophase leptotene |
| OV  | ovarian cavity     |



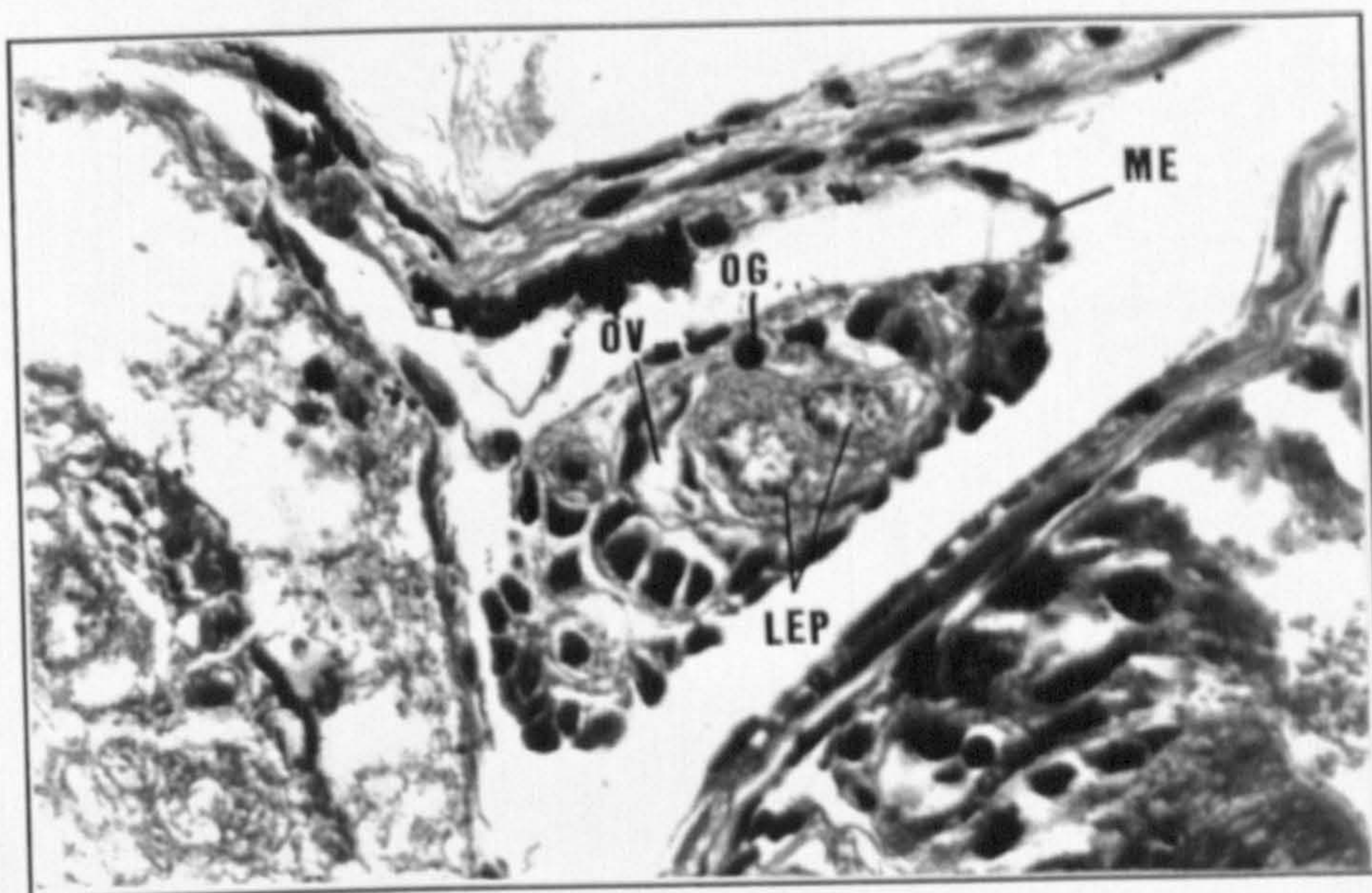


Plate 3.2



Plate 3.3 Transverse sections of *O. niloticus* ovaries.  
Stained with Heidenhain's haematoxylin.

(3.3a) 18 day post-hatched fry x1,000

(3.3b) 21 day post-hatched fry x1,500

OV ovarian cavity

DIP prophase diplotene

NU nucleolus

CH chromatins

OG oogonia

ST.1 stage 1 oocytes



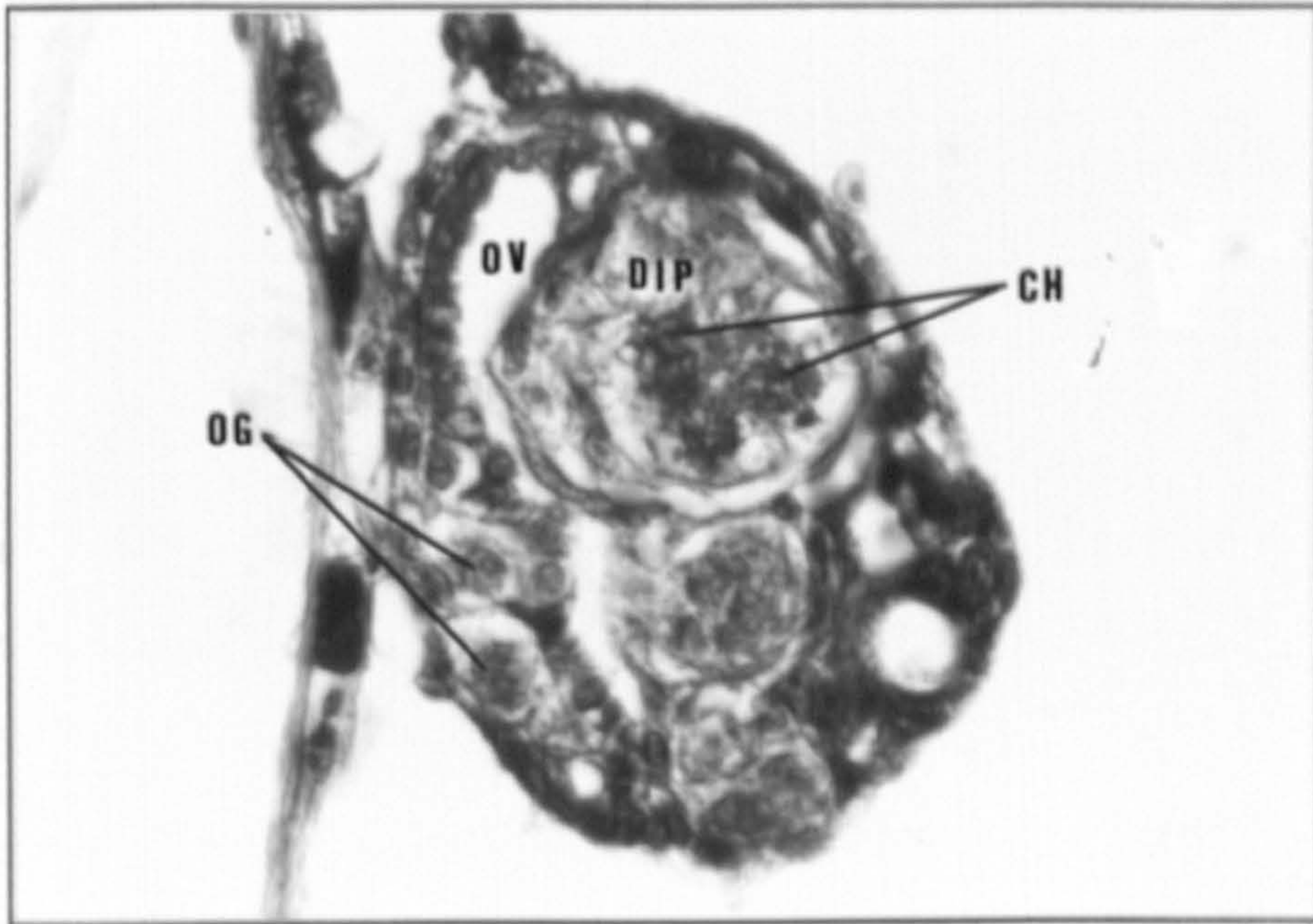


Plate 3.3a

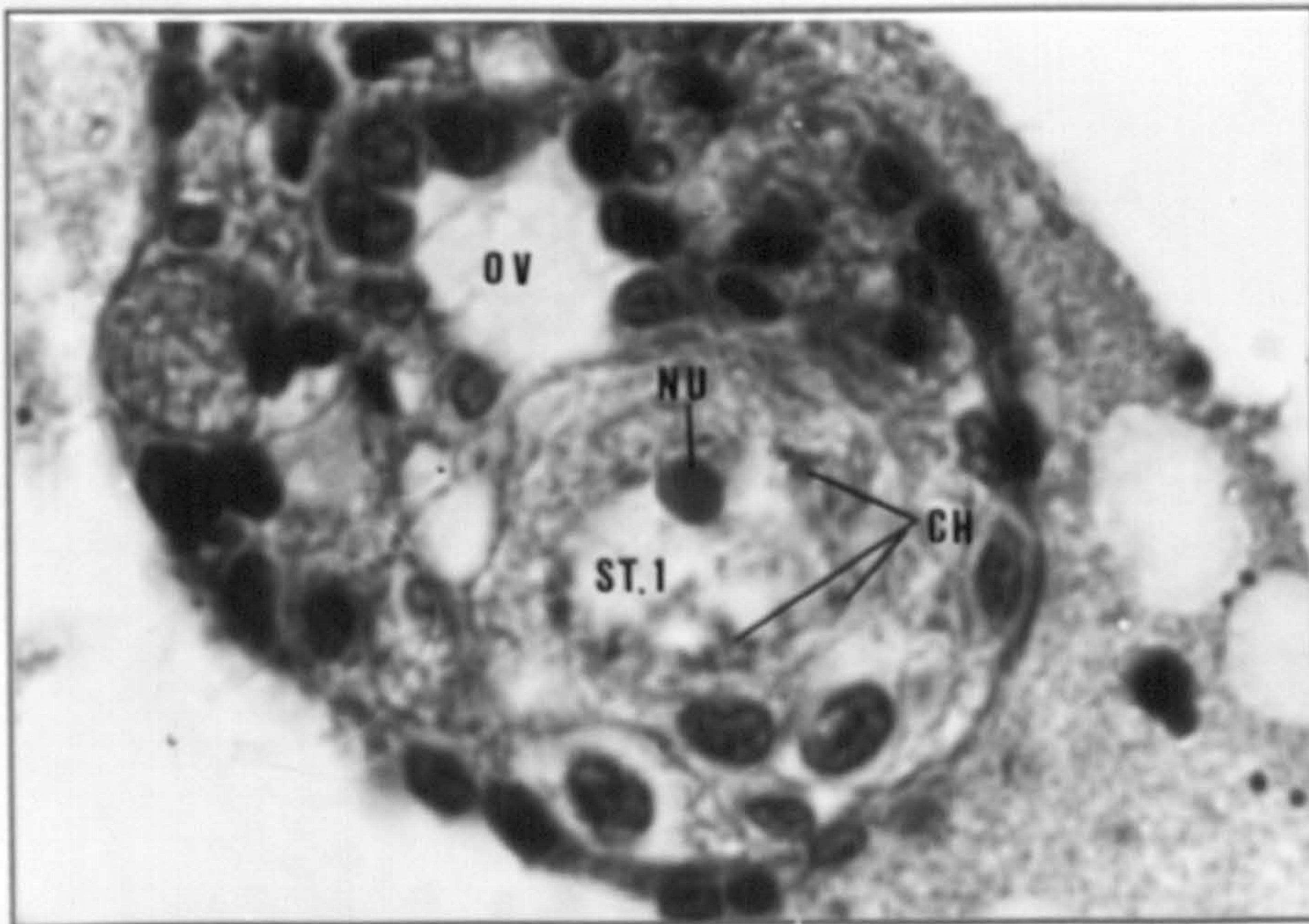


Plate 3.3b

Plate 3.4 Transverse sections of *O. niloticus* ovaries.  
Stained with Heidenhain's haematoxylin.

(3.4a) 28 day post-hatched fry x675

(3.4b) 42 day post-hatched fry x150

OV ovarian cavity

ME mesogonium

B blood vessel

ST.1 stage 1 oocytes

ST.2 stage 2 oocytes



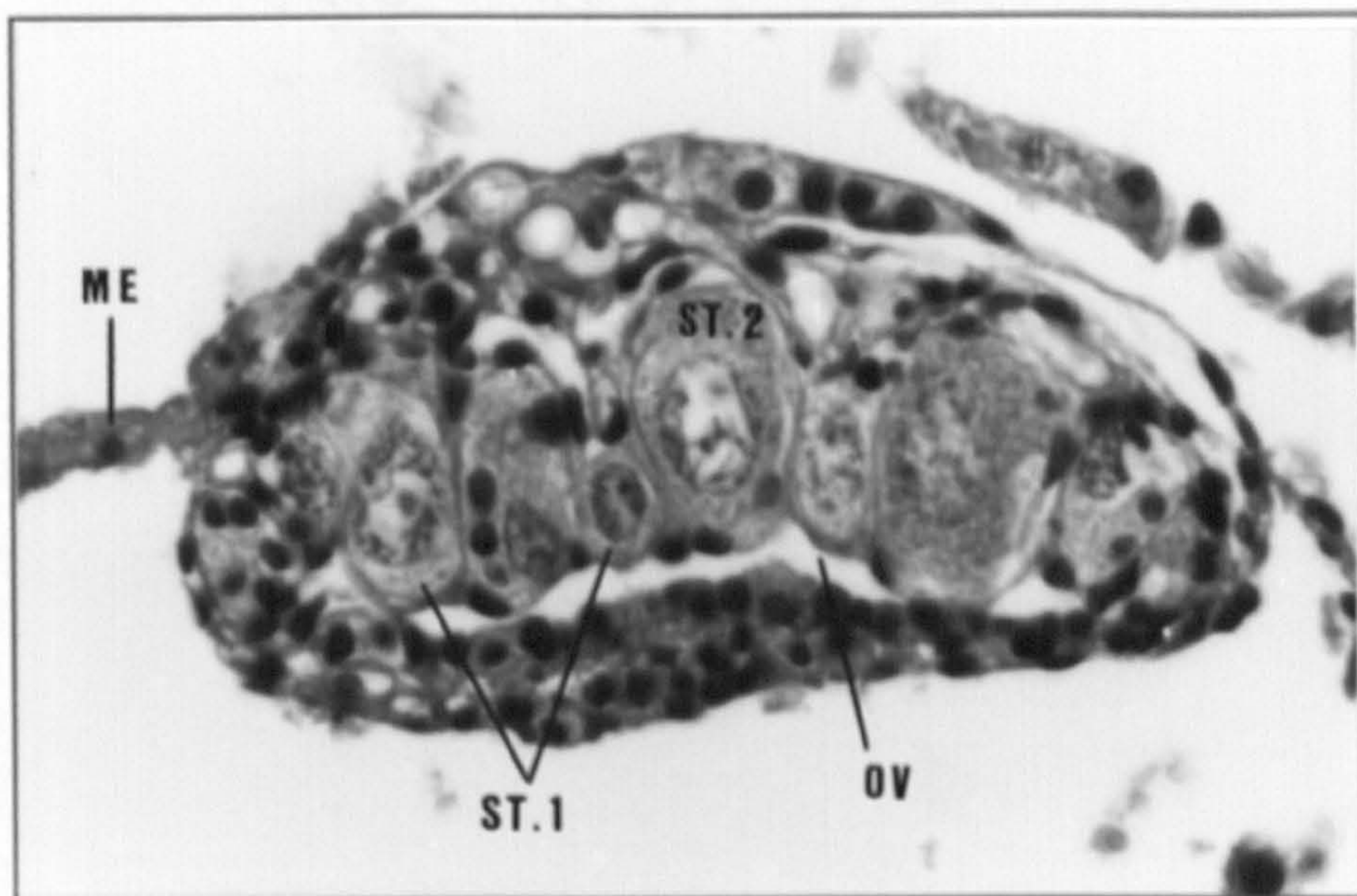


Plate 3.4a

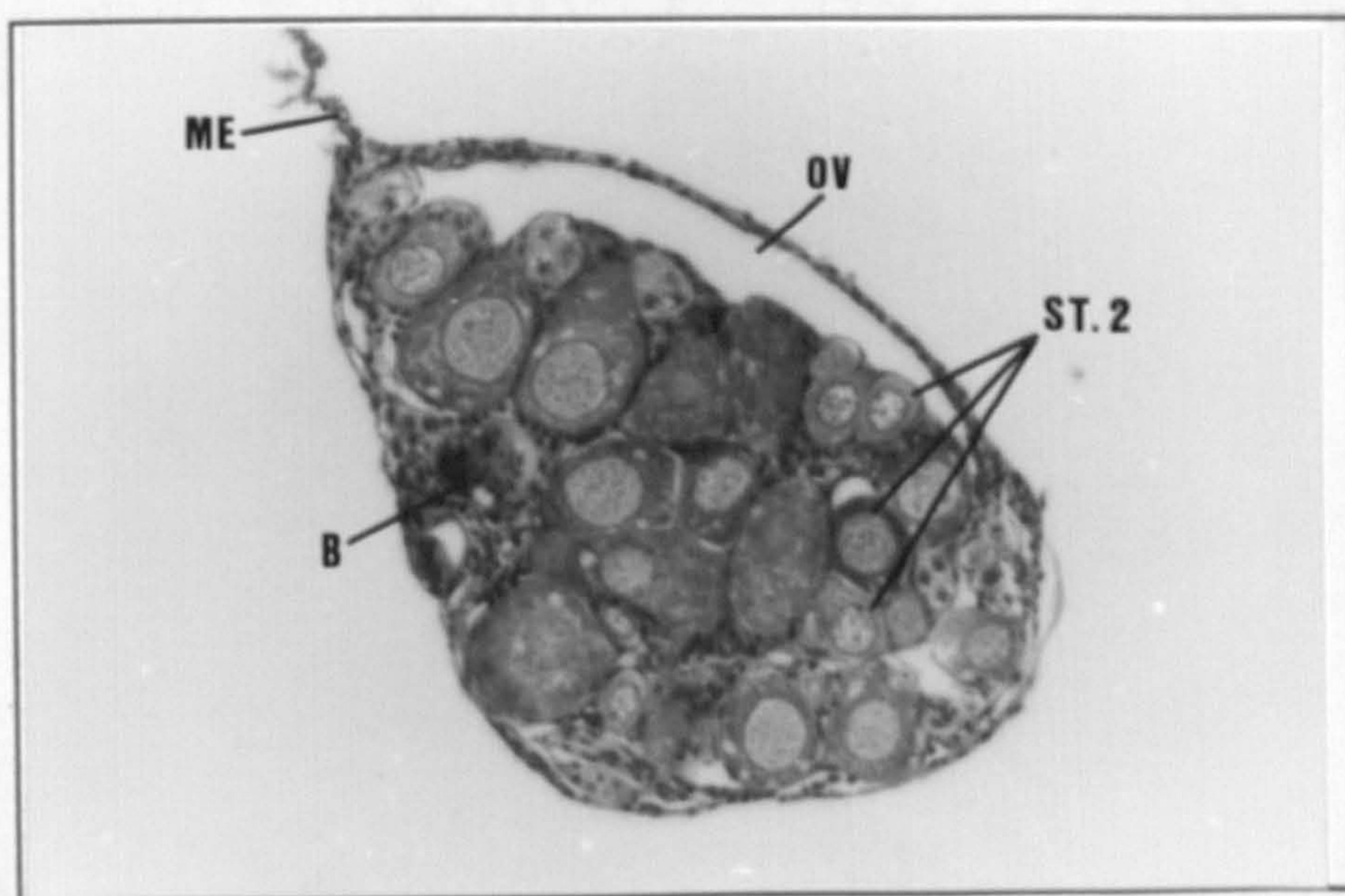


Plate 3.4b



Plate 3.5 Sections of *O. niloticus* ovaries showing stage 2 and stage 3 oocytes. Arrow indicates migration of the Balbiani bodies to the peripheral of oocyte cytoplasm.

(3.5a) Stained with haematoxylin eosin. x150

(3.5b) Stained with polychrome. x300

2A the early of stage 2 oocytes

2B the mid of stage 2 oocytes

2C the end of stage 2 oocytes

ST.3 stage 3 oocytes



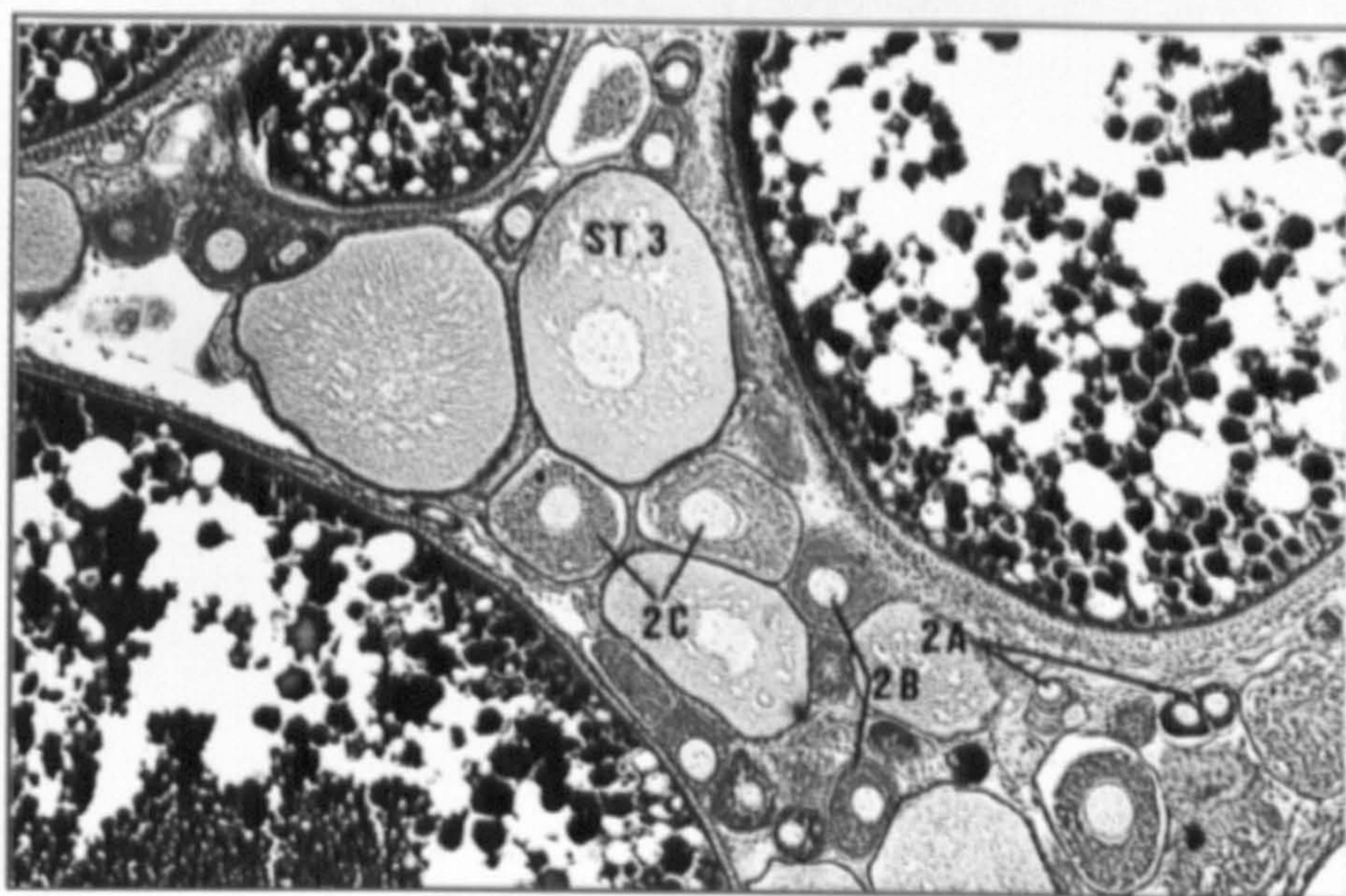


Plate 3.5a

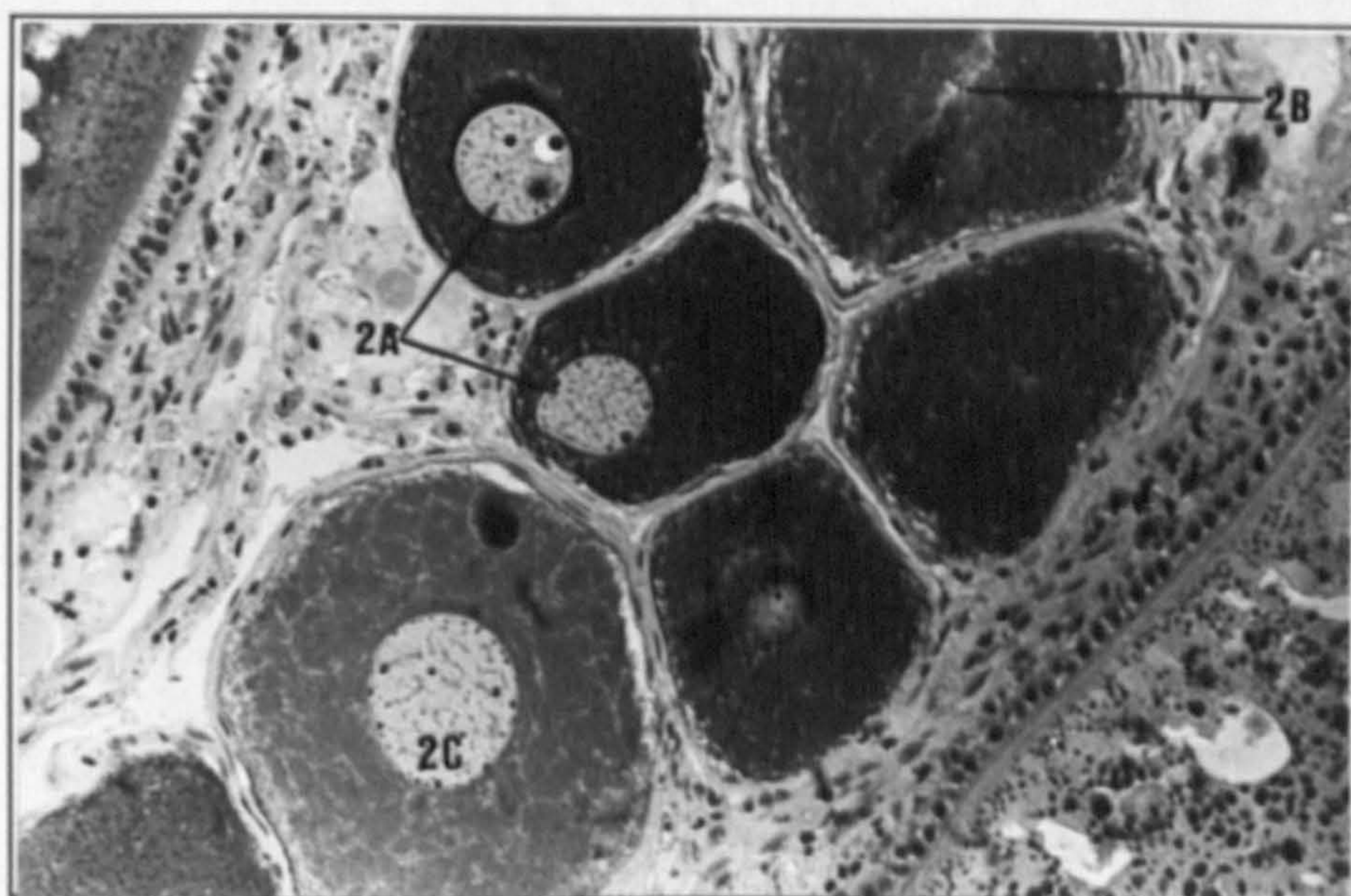


Plate 3.5b



Plate 3.6 Transverse section of *O. niloticus* ovaries showing the different phases of stage 2 oocytes at five days after spawning. Sections stained with polychrome to highlight Balbiani bodies (indicate by arrows)

(3.6a) x 600

(3.6b) x 600

GR granulosa layer  
TH thecal layer  
NE nuclear envelop  
NI nucleoli  
CH chromotins  
2A early stage 2 oocytes  
2B mid stage 2 oocytes  
2C late stage 2 oocytes  
ST.5 stage 5 oocytes



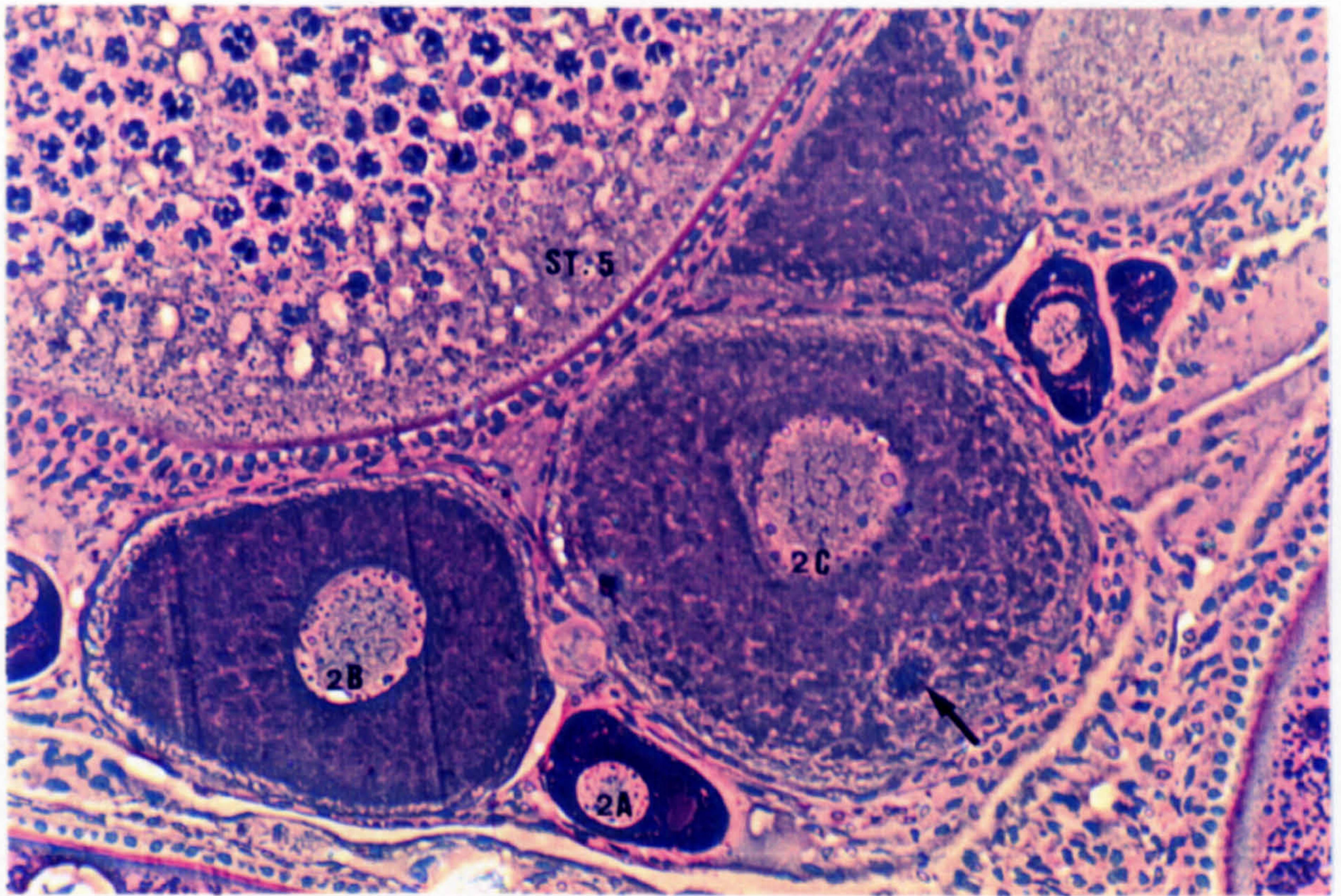


Plate 3.6a

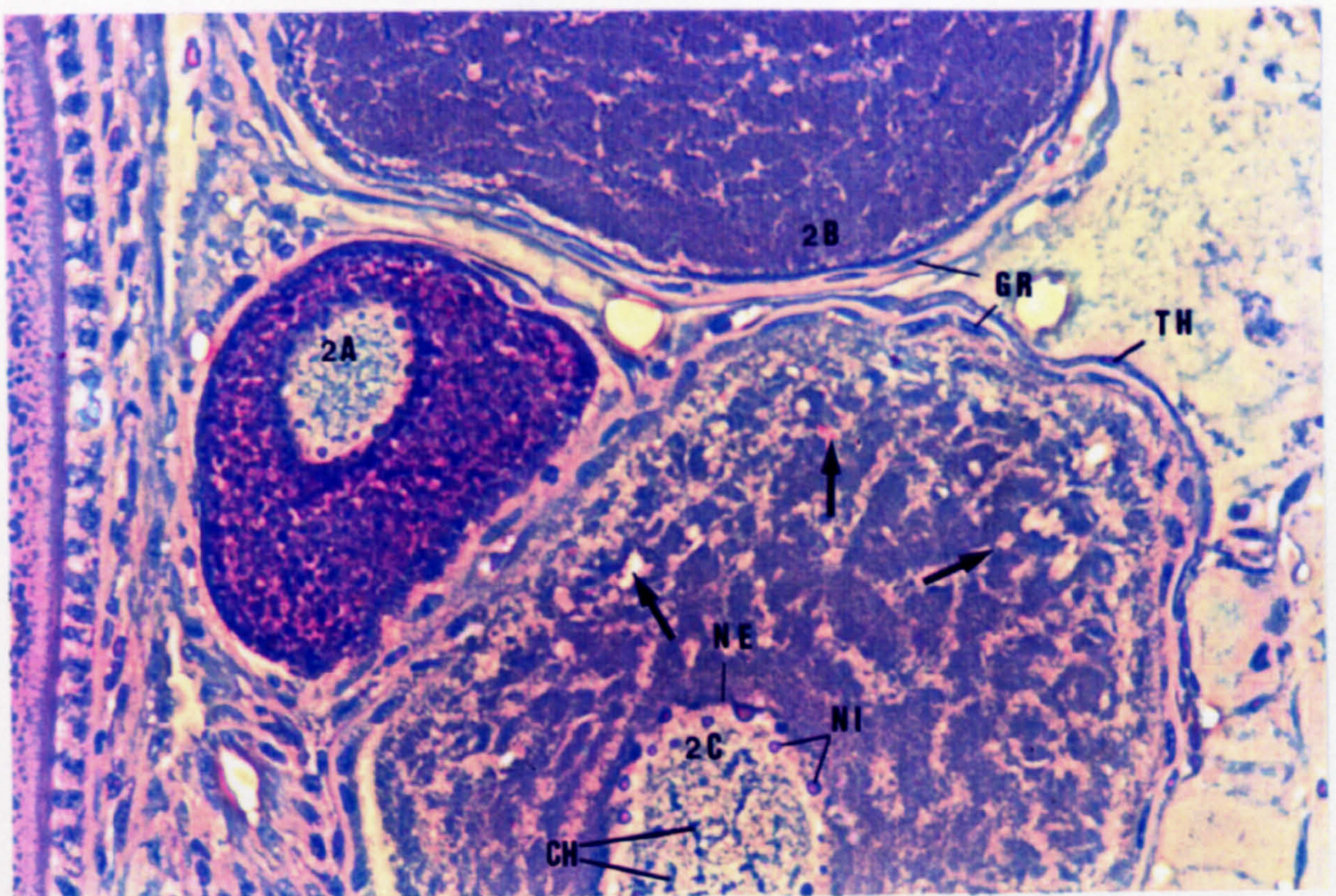


Plate 3.6b



Plate 3.7 Section of 22 weeks *O. niloticus* ovaries. Stained with polychrome. Arrow indicates migration of Balbiani bodies.

(3.7a) x 150

(3.7b) x 300 phase contrast

|      |                   |
|------|-------------------|
| AT.  | atretic oocytes   |
| F    | follicular layers |
| ZP   | zona pellucida    |
| NI   | nucleoli          |
| NE   | nuclear envelop   |
| 2A   | stage 2A oocytes  |
| 2B   | stage 2B oocytes  |
| 2C   | stage 2C oocytes  |
| ST.3 | stage 3 oocytes   |
| ST.4 | stage 4 oocytes   |
| ST.6 | stage 6 oocytes   |



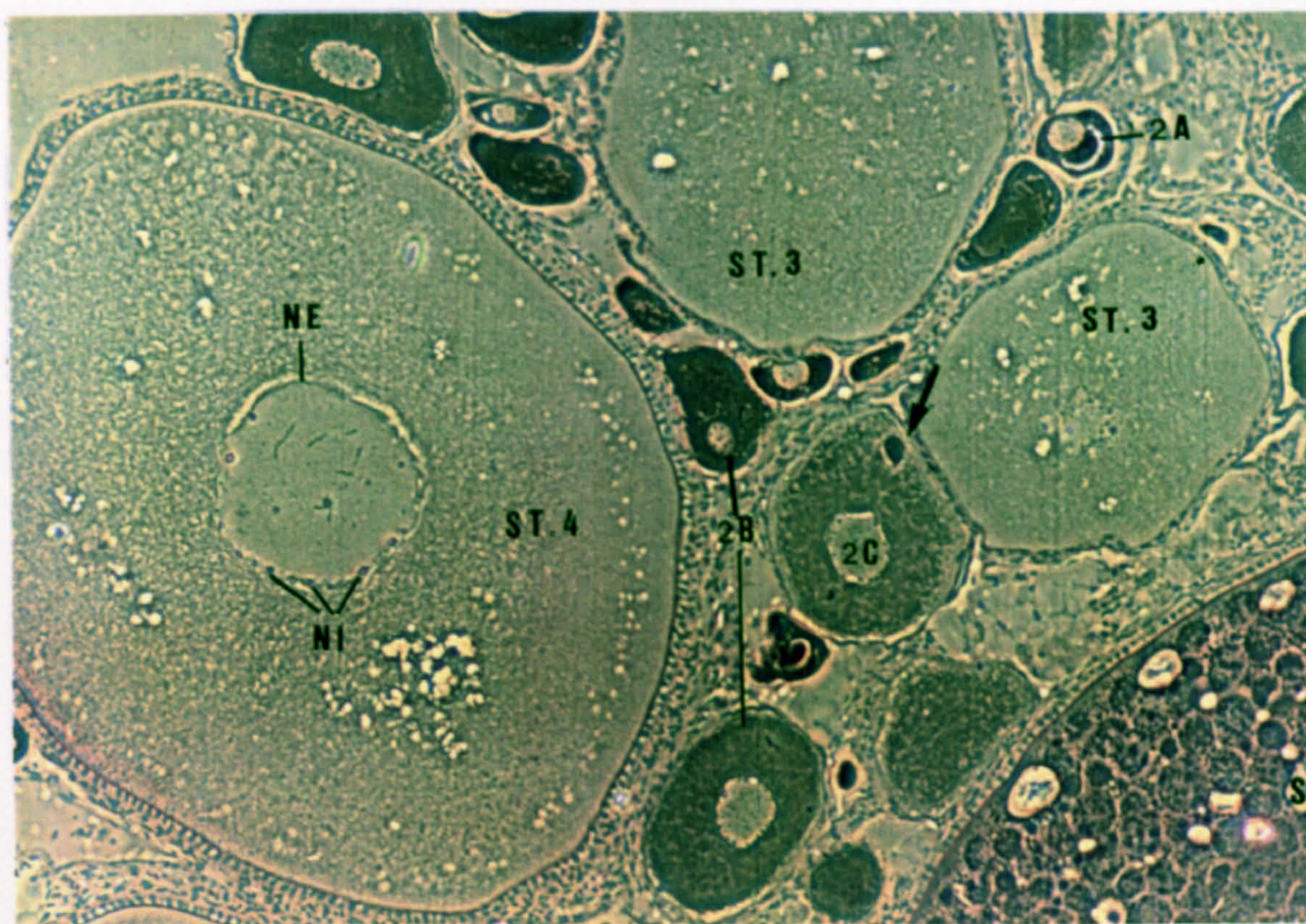
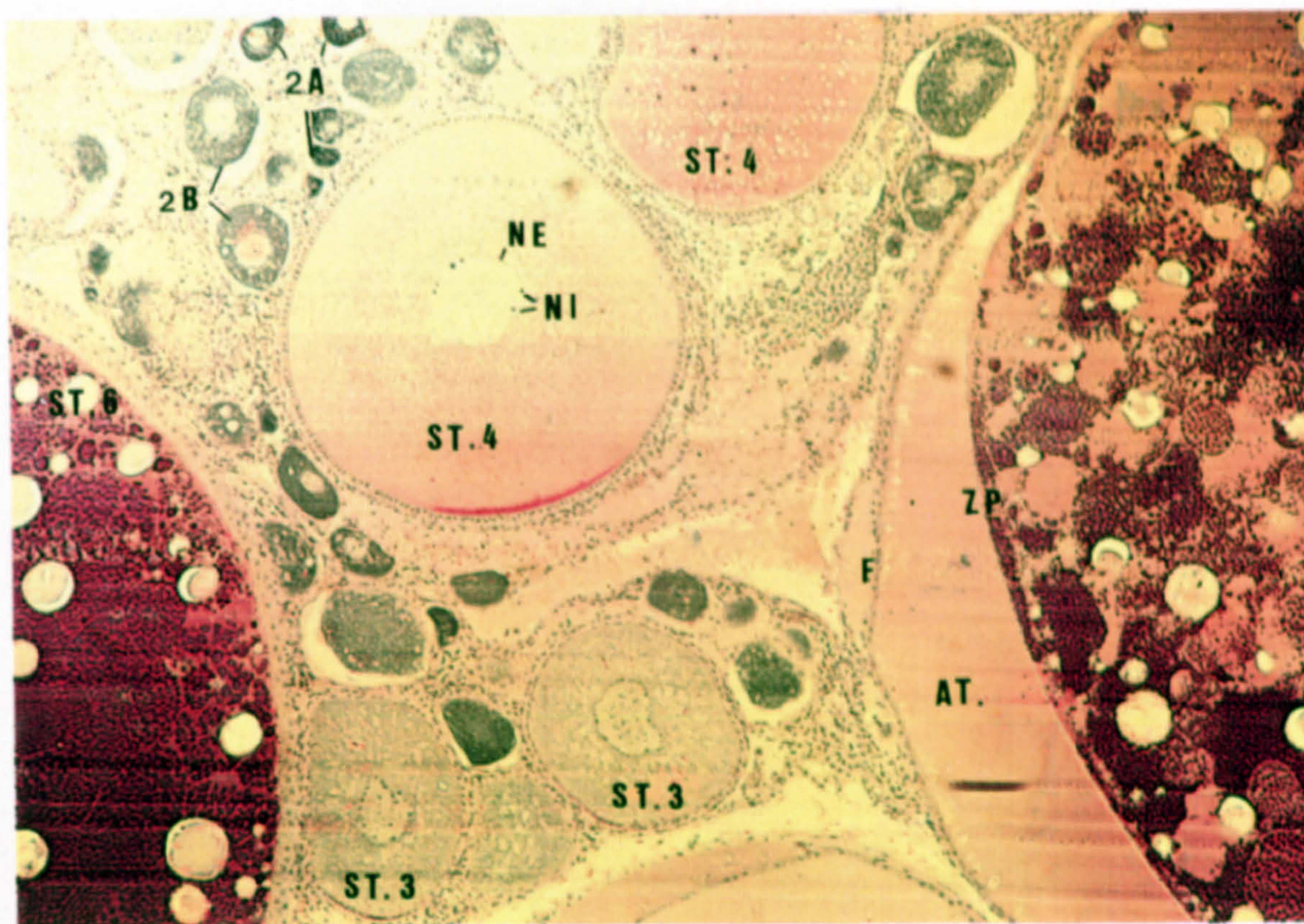




Plate 3.8 Transverse section of 22 weeks *O. niloticus*  
ovaries. Stained with polychrome.

(3.8a) The early stage 5 oocytes x98

(3.8b) The mid stage 5 oocytes x150

|      |                          |
|------|--------------------------|
| F    | follicular layers        |
| ZP   | zona pellucida           |
| YG   | yolk granules            |
| YP   | yolk pellets             |
| NI   | nucleoli                 |
| NE   | nuclear envelop          |
| GR   | granulosa layer          |
| TH   | thecal layer             |
| 2A   | early of stage 2 oocytes |
| ST.5 | stage 5 oocytes          |
| AT.  | atretic oocytes          |



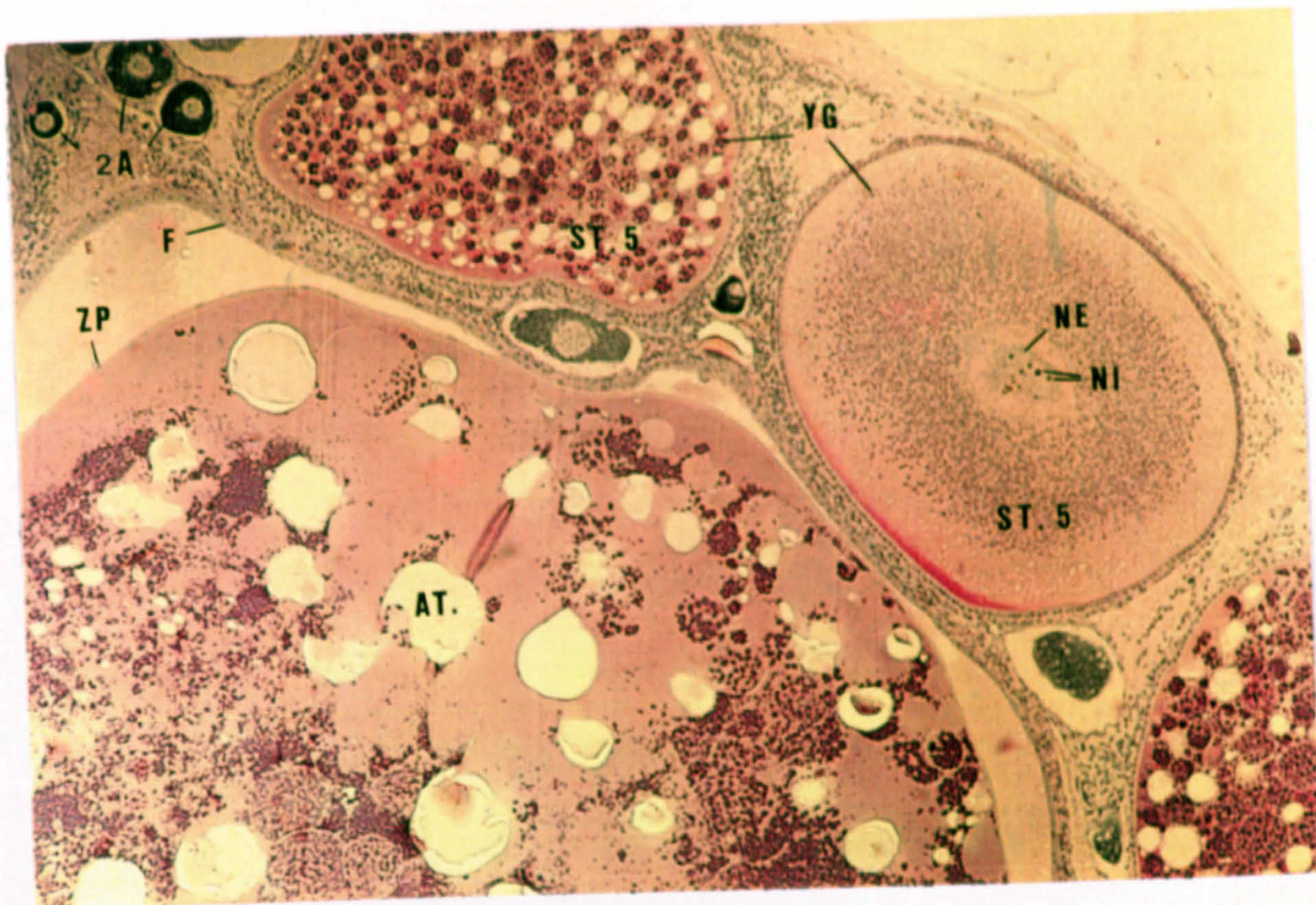


Plate 3.8a

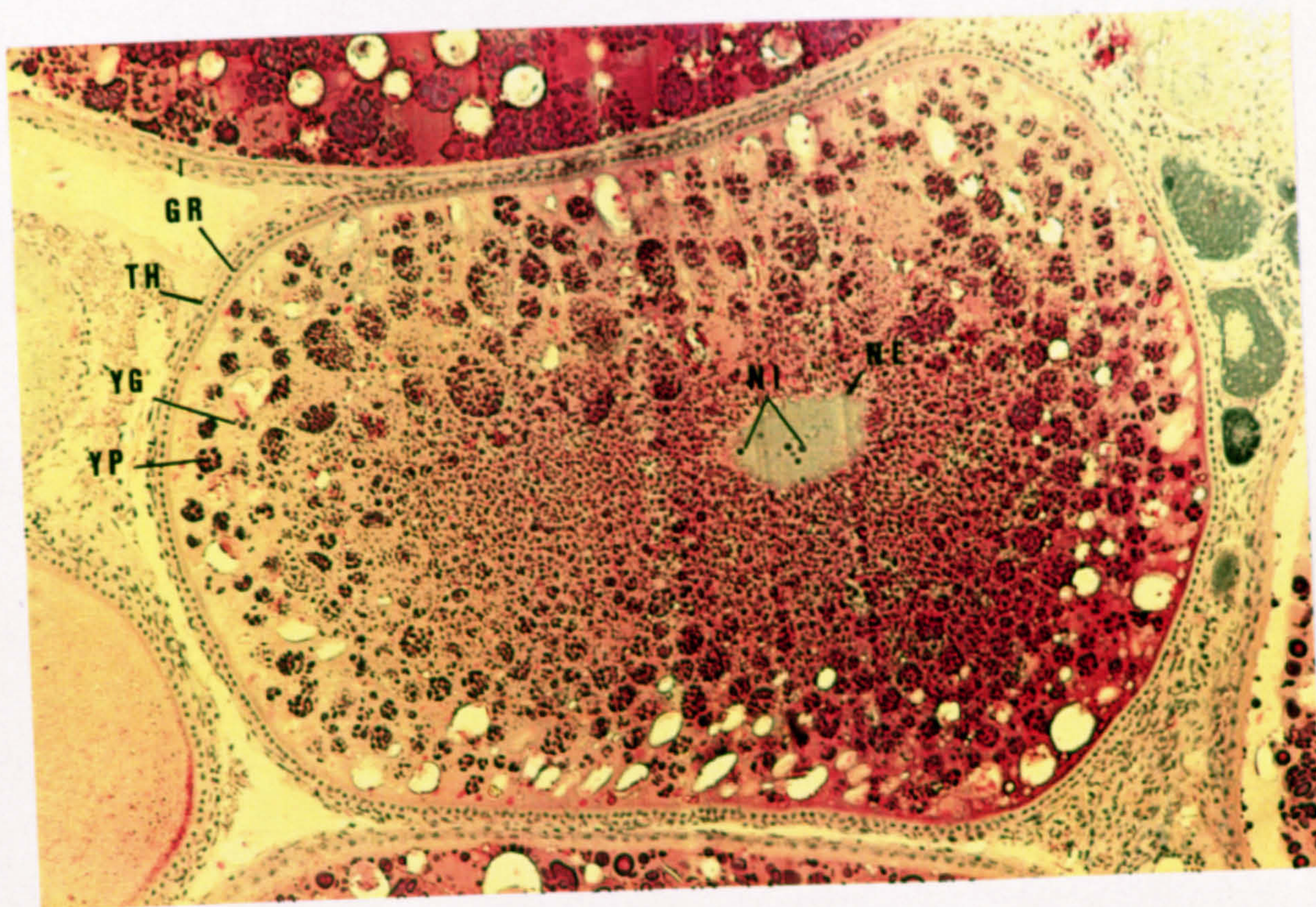


Plate 3.8b



Plate 3.9 Transverse sections of mature stage 6 oocytes of 22 weeks old *O. niloticus*. Showing germinal vesicle migration to animal pole of the oocytes. Stained with polychrome x150

|      |                 |
|------|-----------------|
| GR   | granulosa layer |
| TH   | thecal layer    |
| ZP   | zona pellucida  |
| NI   | nucleoli        |
| NE   | nuclear envelop |
| ST.6 | stage 6 oocyte  |



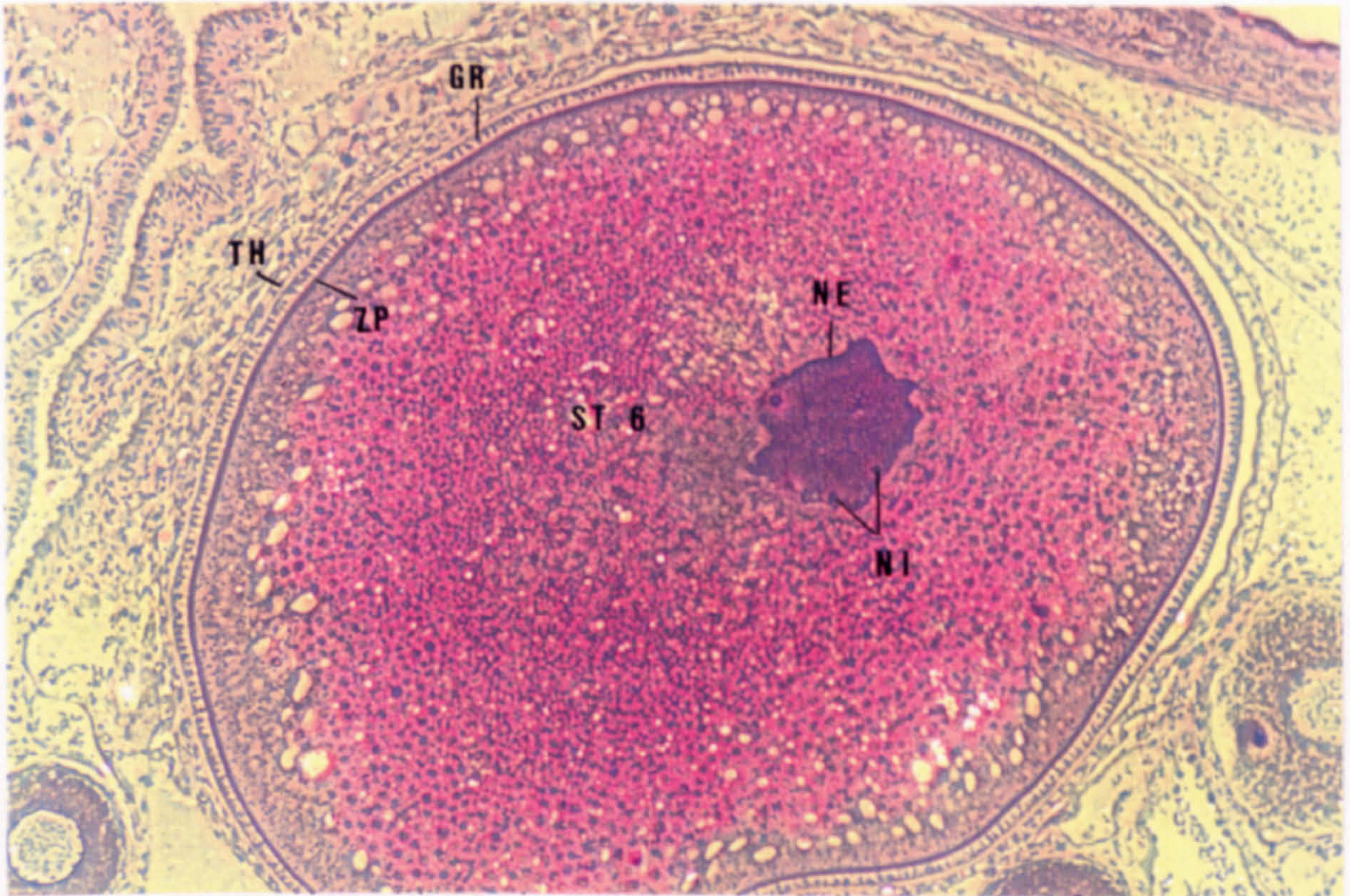


Plate 3.9



Plate 3.10 Transverse sections of *O. niloticus* oocytes in different stages. Comparing follicle cells and layers of stages 2, 3, 4, 5 and 6 oocytes. Sections stained with polychrome.

(3.10a) x600 phase contrast

(3.10b) x600 phase contrast

|      |                          |
|------|--------------------------|
| GR   | granulosa layer          |
| TH   | thecal layer             |
| ZP   | zona pellucida           |
| ZI   | zona interna             |
| ZX   | zona externa             |
| NE   | nuclear envelop          |
| NI   | nucleoli                 |
| 2A   | early of stage 2 oocytes |
| 2B   | mid of stage 2 oocytes   |
| ST.2 | stage 2 oocytes          |
| ST.3 | stage 3 oocytes          |
| ST.4 | stage 4 oocytes          |
| ST.5 | stage 5 oocytes          |
| ST.6 | stage 6 oocytes          |



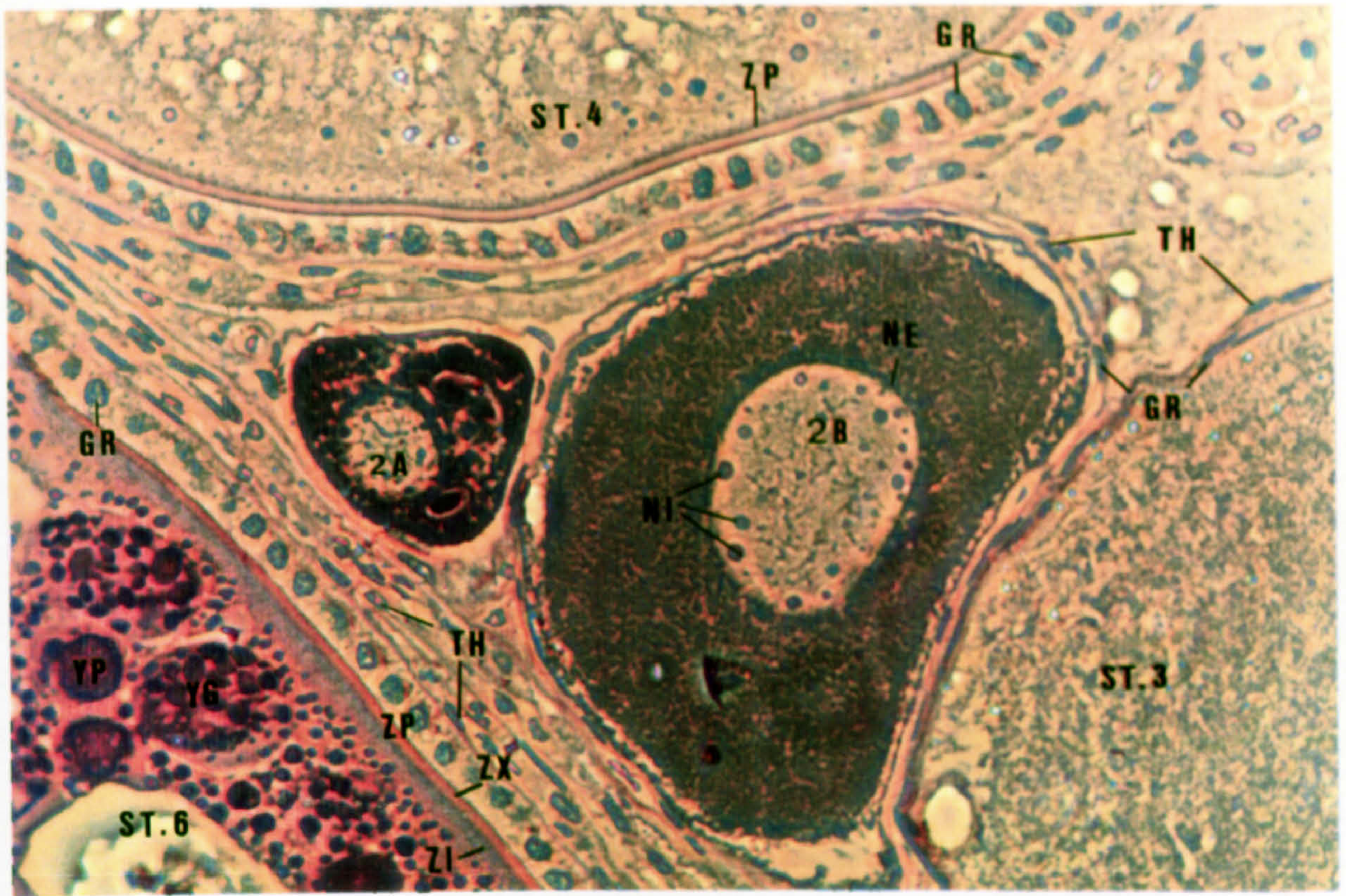


Plate 3.10a

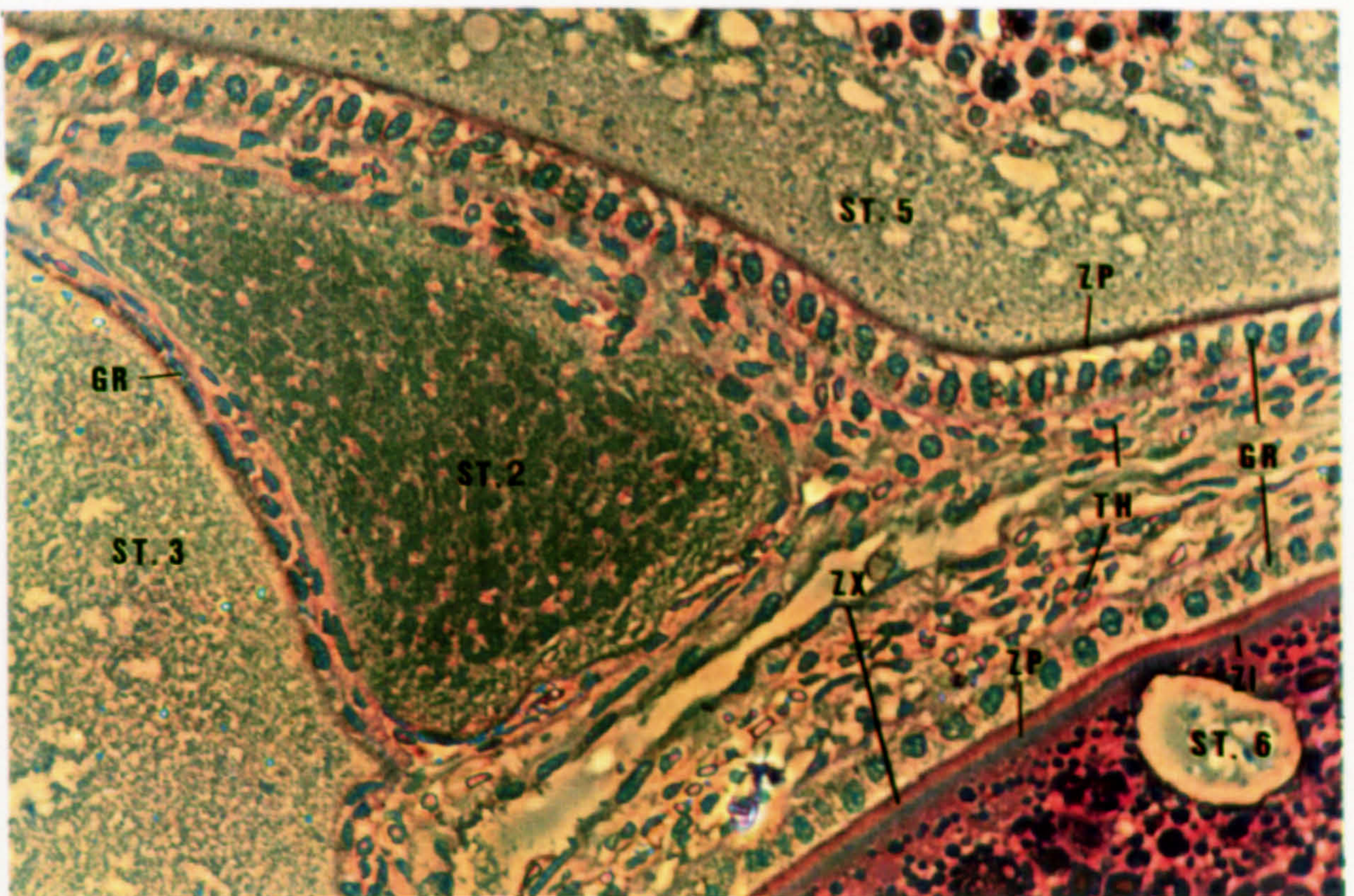


Plate 3.10b



Plate 3.11 Transverse sections of *O. niloticus* ovaries at day one after spawning to compare follicular layers, cytoplasm, yolk pellets in different developmental stage of oocytes. Stained with polychrome.

(3.11a) x650

(3.11b) x1500

|      |                  |
|------|------------------|
| GR   | granulosa layer  |
| TH   | thecal layer     |
| ZP   | zona pellucida   |
| CO   | cortical alveoli |
| YG   | yolk granules    |
| YP   | yolk pellets     |
| ST.3 | stage 3 oocytes  |
| ST.4 | stage 4 oocytes  |
| ST.5 | stage 5 oocytes  |
| ST.6 | stage 6 oocytes  |



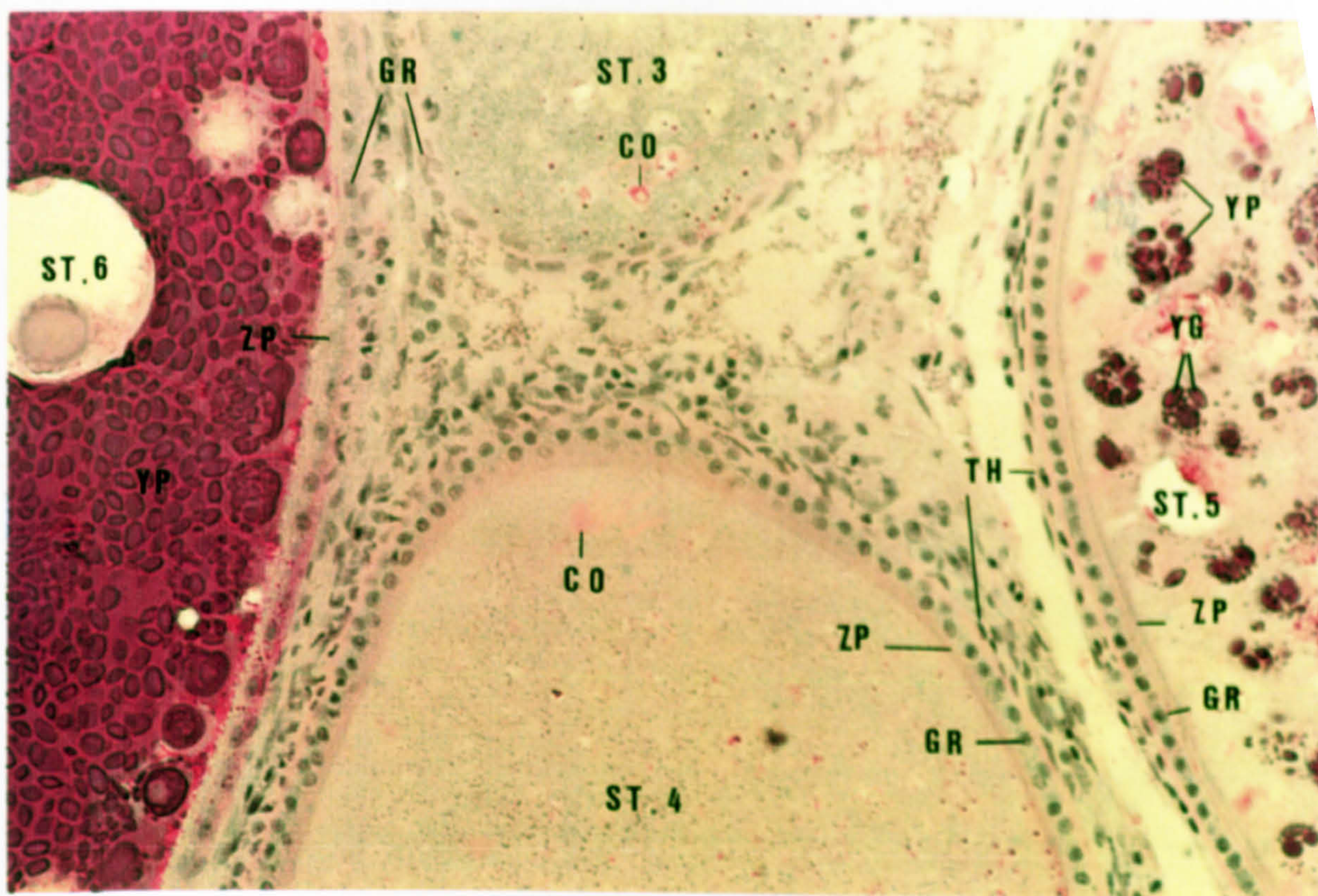


Plate 3.11a

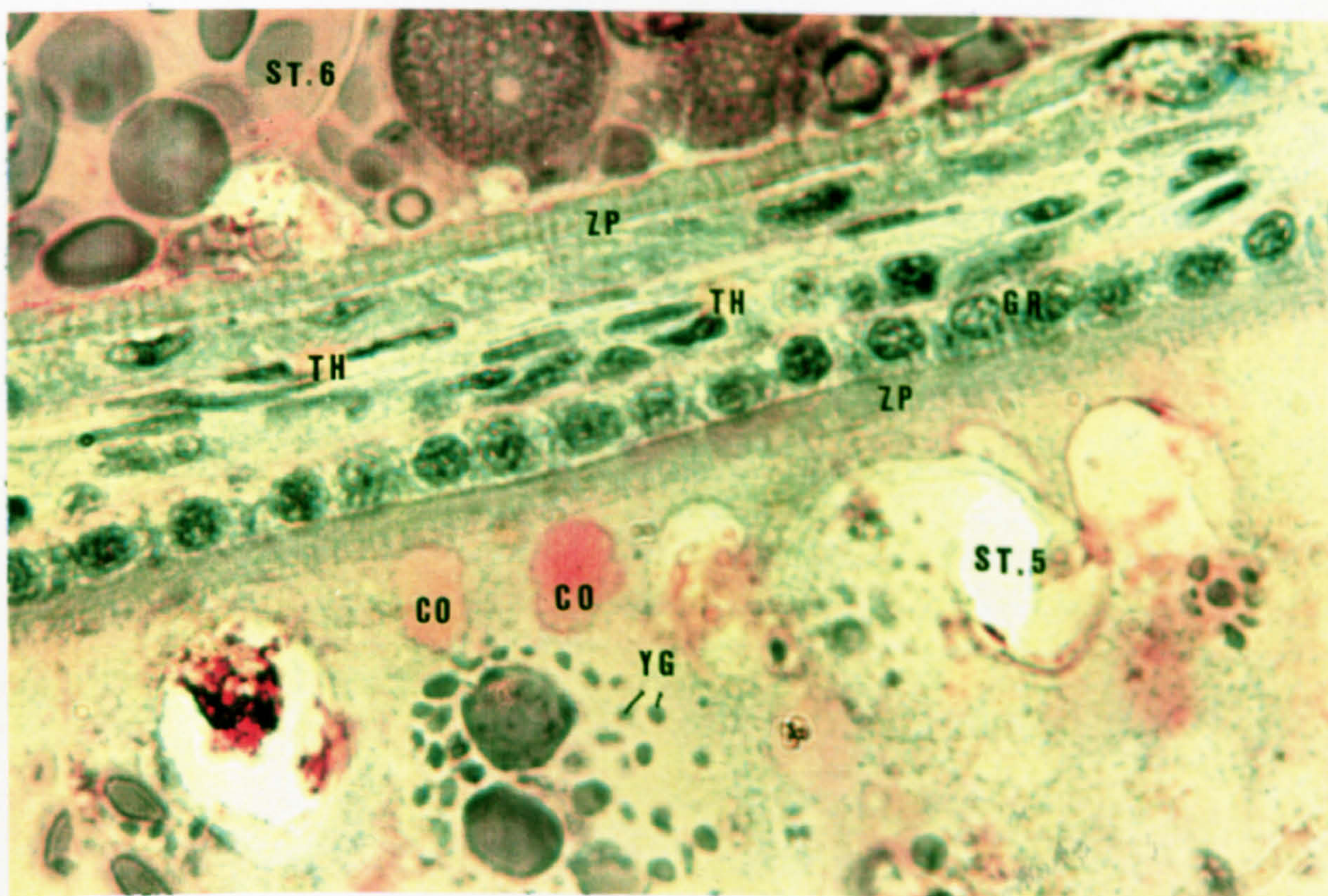


Plate 3.11b



Plate 3.11 Transverse sections of *O. niloticus* ovaries at day one after spawning to compare follicular layers, cytoplasm, yolk pellets in different developmental stage of oocytes. Stained with polychrome.

(3.11a) x650

(3.11b) x1500

|      |                  |
|------|------------------|
| GR   | granulosa layer  |
| TH   | thecal layer     |
| ZP   | zona pellucida   |
| CO   | cortical alveoli |
| YG   | yolk granules    |
| YP   | yolk pellets     |
| ST.3 | stage 3 oocytes  |
| ST.4 | stage 4 oocytes  |
| ST.5 | stage 5 oocytes  |
| ST.6 | stage 6 oocytes  |



Plate 3.12 Transverse sections of *O. niloticus* ovaries on day one after spawning. Sections stained with polychrome to show  $\alpha$  atretic oocytes. Arrows indicate acidophilic yolk fractions.

(3.12a)  $\alpha$  atretic oocytes x60

(3.12b)  $\alpha$  atretic oocytes x60

|     |                          |
|-----|--------------------------|
| F   | follicular layers        |
| PF  | post-ovulatory follicles |
| NU  | nucleus                  |
| AT. | atretic oocyte           |



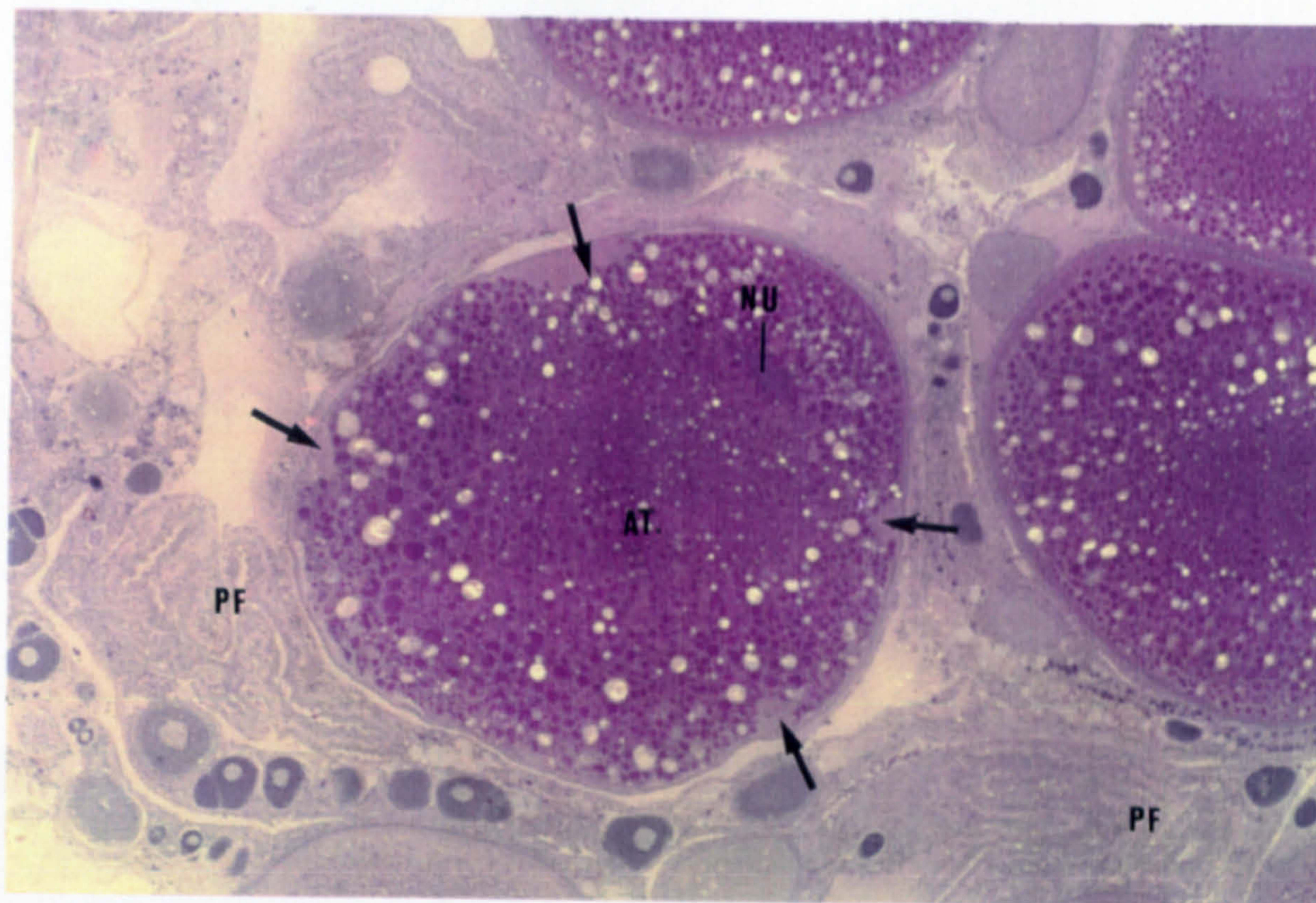


Plate 3.12a

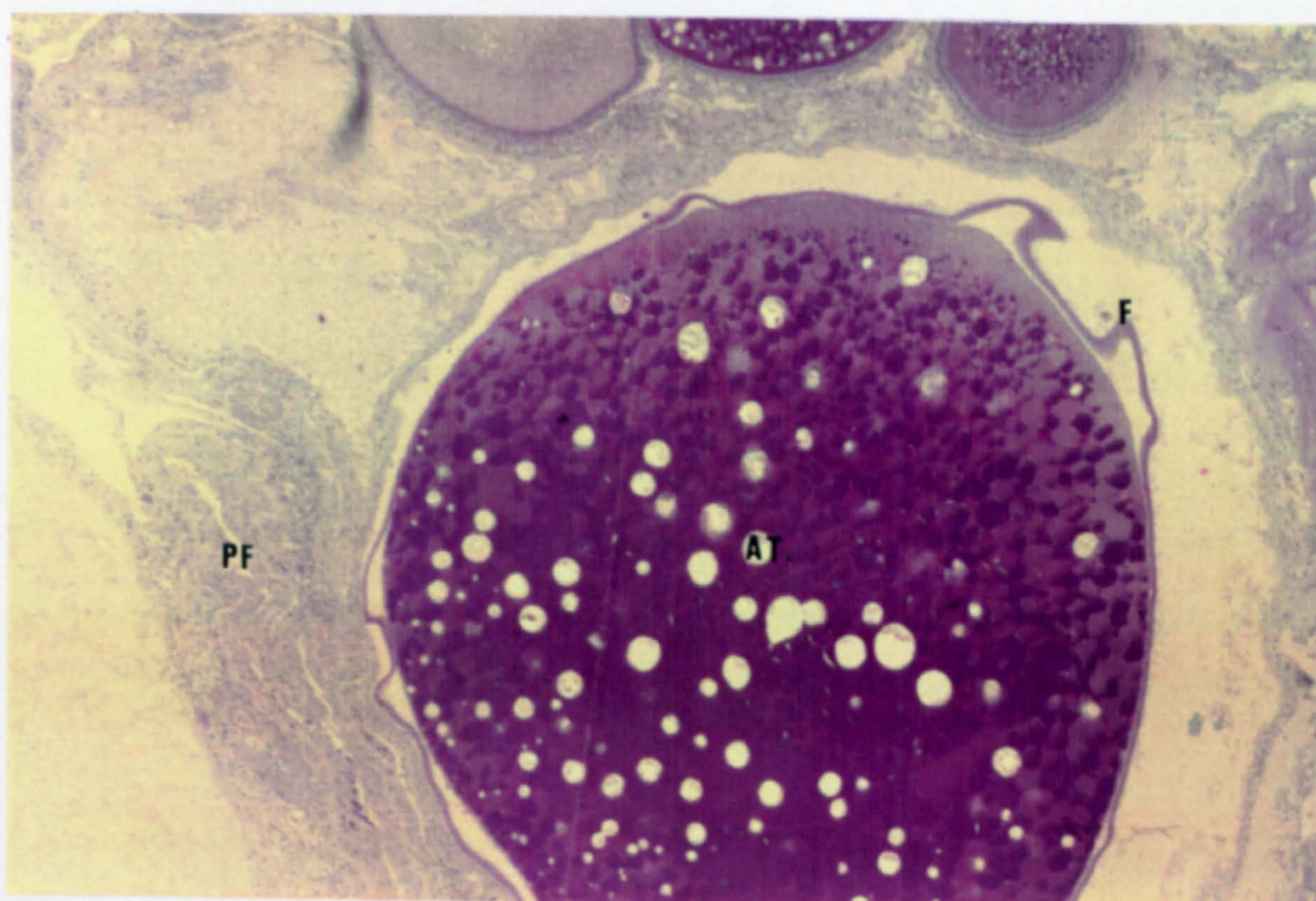


Plate 3.12b



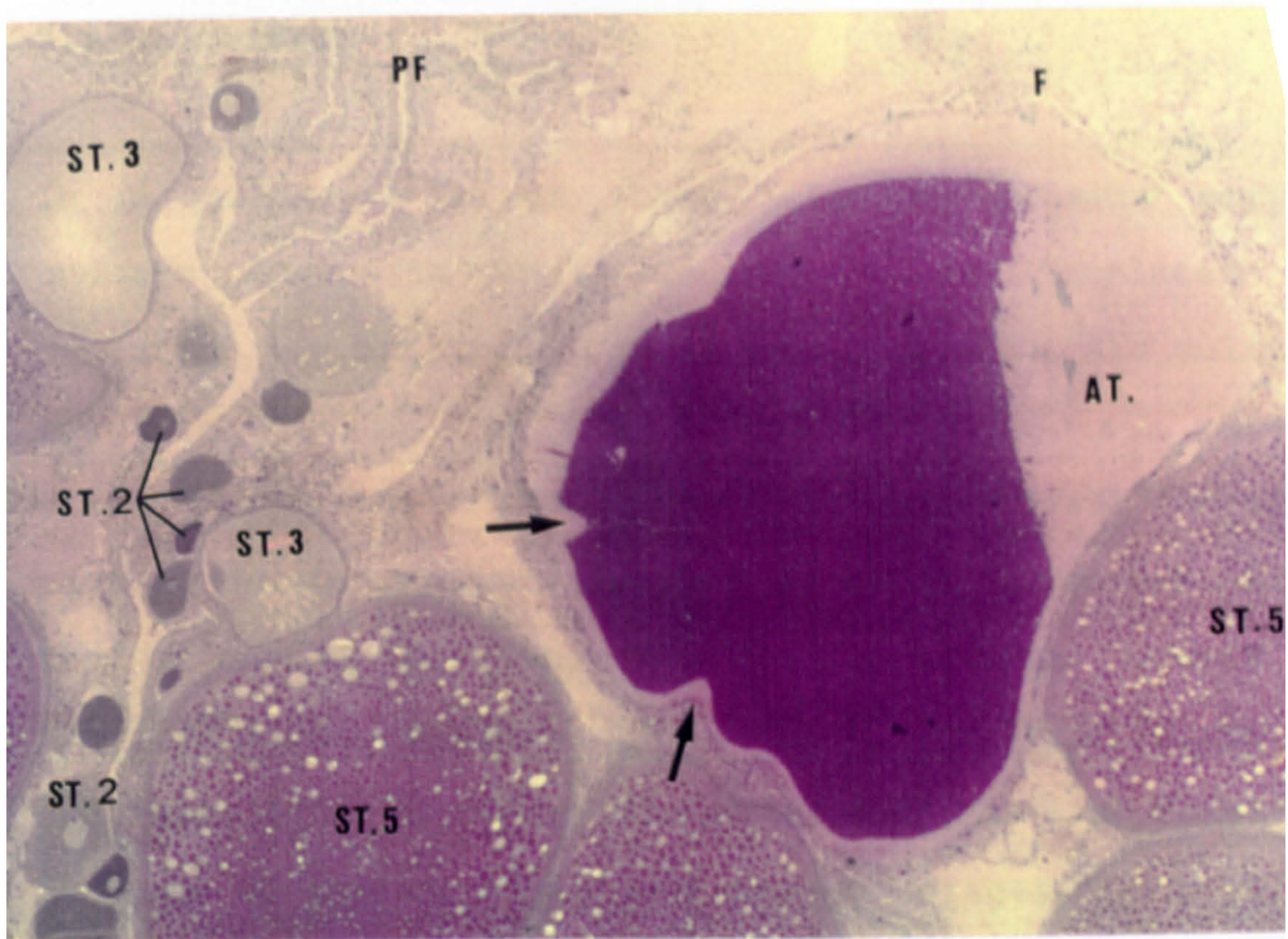
Plate 3.13    Transverse sections of *O. niloticus* ovaries on day one after spawning. Section stained with polychrome to show different atretic oocytes. Arrows indicate invasion of follicles into the oocytes.

(3.13a)  $\beta$  atretic oocyte x60

(3.13b)  $\gamma$  atretic oocyte x60

|      |                         |
|------|-------------------------|
| F    | follicles               |
| PF   | post ovulatory follicle |
| GR   | granulosa layer         |
| TH   | thecal layer            |
| LY   | liquefied yolk          |
| ST.2 | stage 2 oocytes         |
| ST.3 | stage 3 oocytes         |
| ST.5 | stage 5 oocytes         |
| AT.  | atretic oocytes         |





atretic oocytes and post (antral) follicles). The immature oocytes were recruited into the maturing oocyte stage. From this it seems that there are two oocyte stages.

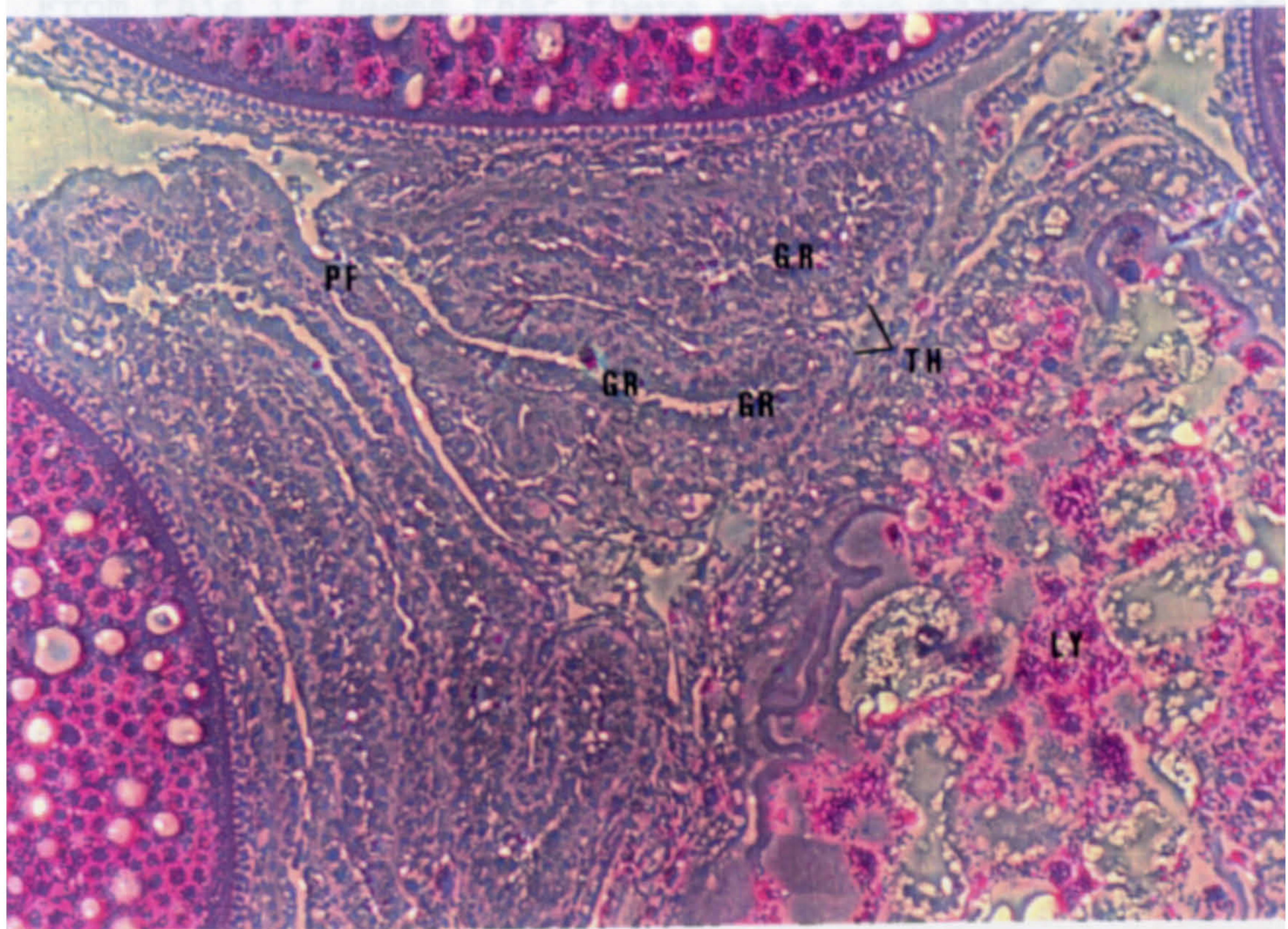


Plate 3.13b



### 3.5 DISCUSSION

In *O. niloticus* as in other teleosts, primary oocytes undergo oogenesis until maturation is completed and ovulation has occurred. *Oreochromis niloticus* are multiple spawners (Dadzie, 1970) and the ovaries contain various sizes and stages of the oocytes. In mature fish, the major part of the ovary was occupied by stage 6 oocytes. Among the stage 6 oocytes, however, a large number of previtellogenic oocytes (stage 2, 3) were also found. In contrast, after spawning, the ovaries of the fish contained a range of oocyte sizes and stages (stages 2, 3, 5, 6, atretic oocytes and post-ovulatory follicles). The immature oocytes were recruited into the maturing oocyte stages. From this it seems that there were two major groups of the oocytes in the ovary of the *O. niloticus*; the previtellogenic and vitellogenic oocytes. Therefore, the ovary of *O. niloticus* may be termed as a "group synchronous ovary" (Wallace et al., 1987).

The oocyte classification had been completed in the present study. Using the polychrome stain, oocyte classification can be accomplished from a single slide.



Although all the ovarian components were highlighted in the present study, oocyte classification was divided into only stage 2, 3, 4, 5, 6, atretic oocytes and post-ovulatory follicles. Stage 1 was not classified due to the size difference when compared to the more advance developmental oocytes and the inability to fully resolve them at fixed or at lower magnification required for stereological analysis. The stages 6 and 7 oocytes were characterized by the position of the nucleus and the break down of the nuclear membrane. These oocytes were very large and their nucleus were very small. Thus, detection of the nuclear position of these two stages was difficult. These two stages were, therefore, combined and categorized as stage 6 oocytes throughout the present study.

Based on the above study, using the polychrome stain, the criteria out lined in Table 3.1 were applied throughout the present study to classify oocyte development in the ovary of *O. niloticus*.



Table 3.1: Criteria for oocyte classification used to discriminate between the different developmental stages in *O. niloticus* ovary using polychrome staining technique. (For details see section 3.4.2)

| Oocyte stages | Comments   |
|---------------|--|
| Stage 2       | Dark blue-black at stage 2a<br>Blue or grey at stage 2b<br>Light blue or grey at stage 2c                          |
| Stage 3       | The absence of the blue or grey cytoplasm and bigger oocytes than 2c   |
| Stage 4       | Pink-staining cytoplasm (PAS+)   |
| Stage 5       | Accumulation of yolk granules, stained with mauve blue and centrally positioned nucleus                            |
| Stage 6       | Formation and fusion of yolk granules and yolk pellets as well as vacuoles in the peripheral cytoplasm             |
| Atretic       | Break down of the nuclear membrane, liquefied yolk, pink yolk mass and brown or black bodies in the remaining yolk |



Table 3.1: Criteria for oocyte classification used to discriminate between the different developmental stages in *O. niloticus* ovary using polychrome staining technique. (For details see section 3.4.2)

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| Atretic       | Break down of the nuclear membrane, liquefied yolk, pink yolk mass and brown or black bodies in the remaining yolk |



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# CHAPTER 4

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#### 4. METHODS FOR ASSESSING OOCYTE NUMBERS AND MATURITY OF *Oreochromis niloticus* (L.)

##### 4.1 INTRODUCTION

The estimation of ovulated eggs by either direct count or indirect mass methods is the most common method for estimating egg numbers (Blay, 1981; Burd and Howlette, 1974). This method, however, excludes small or unovulated eggs and therefore, the number of eggs can be underestimated.

The estimated number of oocytes is used to determine the fecundity of the fish. If the number of mature or ripe oocytes in the ovary are estimated, the fecundity of fish may be easily obtained on the assumption that mainly ripe oocytes are shed from the fish at each spawning time. Therefore, ripe oocyte number may be used to estimate fecundity of fish.

The accurate determination of oocyte development depends on the method used for staging oocytes (West, 1990). Generally, the external appearance of the ovary (e.g., big or small ovary) and colour of oocytes (e.g., white, yellow, green or grey) are simple methods which are rapid but have a low level of accuracy. Alternatively, the proportion of the ovary to fish body weight or the gonadosomatic index (GSI) is commonly used to estimate oocyte development or maturity of fishes. Oocyte sizes may also be used to predict the



stages of oocytes which are obtained from Gilson's fluid method. This oocyte staging, however, requires the oocyte diameters corresponding to different stages to be determined from histological sections. The staging of oocytes from histological section has been reported to be the most accurate method since the oocytes are classified according to their histo-morphological structure but it is expensive and time consuming (West, 1990). The merits of the different methods that can be used to estimate oocyte numbers are described below.

#### 4.1.1 Gilson's Fluid Method

This technique is based on the digestion of ovarian tissue in Gilson's fluid over a period of 4 - 6 weeks (in *O. niloticus*) to 4 months (in herring, sole, mackerel; Emerson, Greer Walker and Witthames, 1990) to free the oocytes from their surrounding tissue. The oocyte numbers are then estimated by either the direct count of the oocyte numbers (Blay, 1981), their subsample weight (Burd and Howlette, 1974) or the subsample volume (Simpson, 1951). Each of the above options is time consuming due to the large numbers of oocytes and size ranges of the different oocyte stages in the ovary.

The Gilson's method has many drawbacks. The solution contains highly toxic chemicals such as mercuric chloride. The digestion process requires a long period and needs



frequent shaking. This method also needs histological preparation for staging of oocytes into different stages according to their diameters.

#### 4.1.2 Stereological Methods

All the stereological methods are based on the Delesse Principle, which was derived in 1847. This fundamental principle states that a random 2-dimensional section can be used to quantify the composition of a 3-dimensional object.

These stereological methods have been successfully applied to estimate oocyte numbers of fish from histological sections of ovaries (Emerson et al., 1990). They are also more accurate for oocyte staging but it requires expensive equipments, e.g., tissue processor, microtome, microscope, etc.

The Delesse Principle can be easily proved. If a cube is placed into an x,y,z coordinate system and is sliced in parallel to the x,z-plane into very thin slices having a thickness  $d_y$  (Figure 4.1). Total sectional area ( $A_{st}$ ) of each slide and sectional object area ( $A_{so}$ ) of interest evidently contain a certain volume of the sectional object ( $V_{so}$ ) and the total volume of the section ( $V_{st}$ ) as shown in the equations below:

$$V_{so} = A_{so} \cdot d_y \quad (1)$$

$$V_{st} = A_{st} \cdot d_y \quad (2)$$



The total sectional volumes ( $V_{st}$ ) and the total sectional object volumes ( $V_{so}$ ) are summed to obtain total volumes of the cube and the object. The total object volume ( $\Sigma V_{so}$ ) is divided by the total section volume ( $\Sigma V_{st}$ ) in equation 3. The volumes of  $V_{so}$  and  $V_{st}$  are replaced with the equation 1 and 2 into equation 4. The equation 4 therefore shows the equality of  $V_{vo}$  and  $A_{ao}$  in equation 5.

$$\frac{\Sigma V_{so}}{\Sigma V_{st}} = \frac{V_{so}}{V_{st}} = V_{vo} \quad (3)$$

$$\frac{\Sigma (A_{so} \cdot d_y)}{\Sigma (A_{st} \cdot d_y)} = \frac{(d_y \Sigma A_{so})}{(d_y \Sigma A_{st})} = \frac{A_{so}}{A_{st}} = A_{ao} \quad (4)$$

$$V_{vo} = A_{ao} \quad (5)$$

In addition, if  $A_{ao}$  is measured by the point counting technique ( $P_{po}$ ), which uses a random grid point procedure (Weibel, 1979) to measure area fraction of an object ( $P_{so}$ ) within a total area ( $P_{st}$ ) of the section then

$$P_{po} = \frac{P_{so}}{P_{st}} \quad (6)$$

Then, we can conclude that :

$$V_{vo} = A_{ao} = P_{po} \quad (7)$$



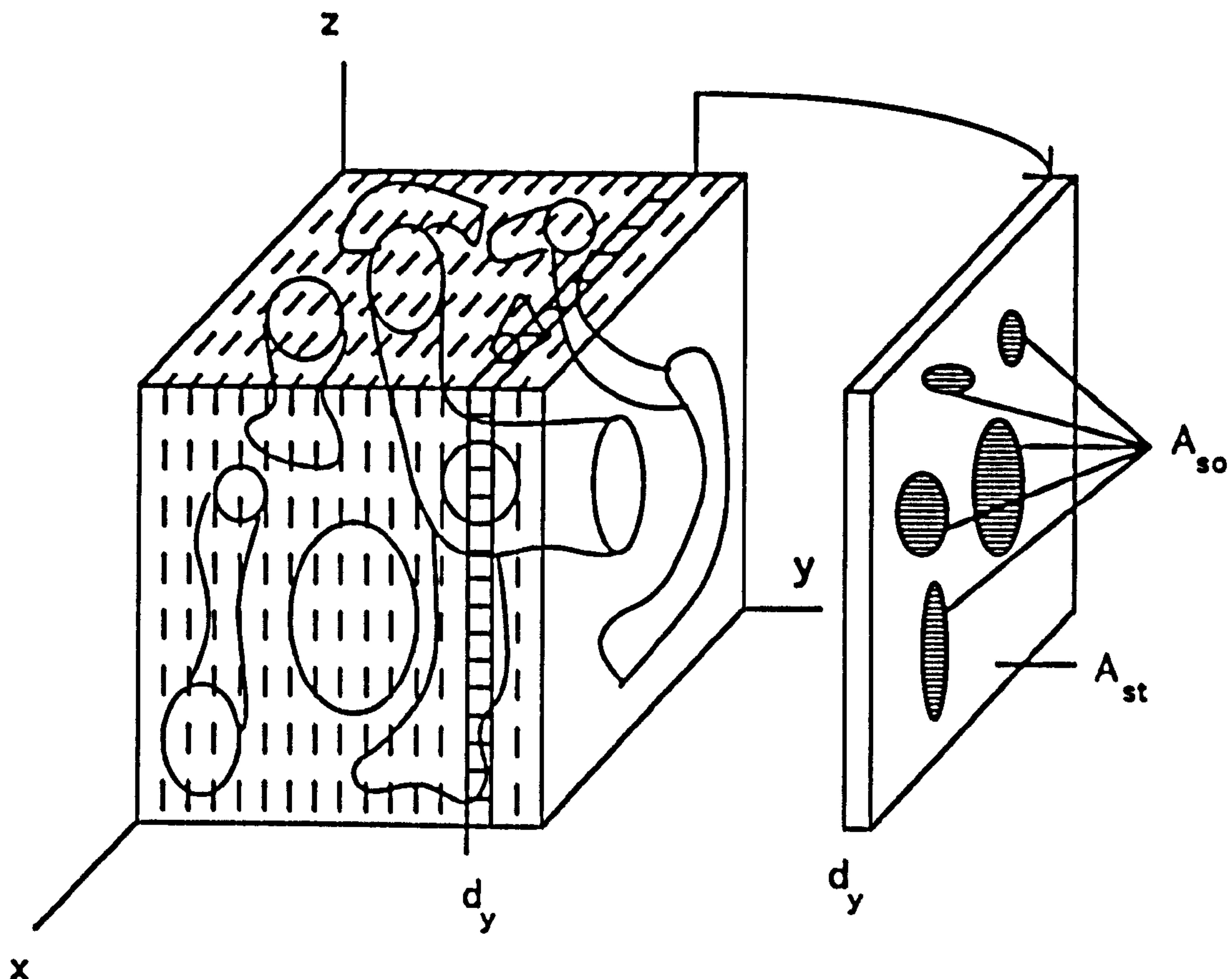


Figure 4.1: Model illustrating the Delesse principle. The sample cube is placed into an  $x, y, z$  coordinate system and sliced parallel to the  $x, z$ -plane into a thickness of  $d_y$ . The  $A_{so}$  is the sectional object area and  $A_{st}$  is the total sectional area. The area fraction ( $A_{so}$ ) and volume fraction ( $V_v$ ) of the object are computed by  $A_{so} = V_v \cdot d_y = A_{so}/A_{st}$ . (Modified from Weibel, 1979).



The stereological techniques outlined below which used the above Delesse Principle can be applied to quantify the area or volume fraction occupied by the objects of interest such as nucleus in a cell, mitochondria in a tissue, oocytes in an ovary, etc. In fish, these methods could be applied to facilitate the enumeration of oocyte numbers from histological sections of ovaries.

#### 4.1.2.1. Planimetric method

This is the simplest of the stereological techniques (Weibel, 1979; 1980). The method directly estimates the area fraction of oocytes in stage  $x$  ( $A_{ax} = V_{vx}$ ) by drawing the outline of stage  $x$  oocytes on graph paper and counting the numbers of square which are occupied by those oocytes. Alternatively, the stage  $x$  oocytes can be traced on to a piece of paper or a plastic sheet which is then cut out and weighed (Weibel, 1979). The weight of the stage  $x$  oocytes is subsequently converted to the area of stage  $x$  oocyte ( $A_{ax}$ ) and then divided by total sectional area of ovary ( $A_{tx}$ ) to obtain area fraction of stage  $x$  oocytes ( $A_{ax} = V_{vx}$ ). Although these are simple techniques, they are the most time consuming of options (Weibel, 1979).



#### 4.1.2.2. Point counting method

This method was developed to be used with a random grid (e.g., double lattice, multipurpose; M168, etc) for measuring the oocyte area occupied by the stage x oocytes ( $A_{sx}$ ). The area fraction of stage x oocytes ( $A_{sx}$ ) can be easily estimated by dividing the number of grid points that overlay the stage x oocytes by the numbers of grid points occupying total sectional area of ovary ( $A_{tx}$ ). This method yields similar results to the planimetric method but requires less time (Weibel, 1979).

#### 4.1.2.3. Numerical density method

Weibel and Gomez (1962) have developed a numerical density equation, to quantify stage x oocyte numbers in a unit volume ( $N_{sx}$ ) from the  $A_{sx}$  or  $V_{vx}$ . The quantity of oocyte needs to be computed from the shape ( $\beta_x$ ) and diameter distribution ( $K_x$ ) of stage x oocytes. Finally the number of stage x oocytes in a unit volume ( $N_{sx}$ ) is multiplied with the total volume of the whole ovary as shown in these following equations:

$$\beta_x = \frac{l_x}{s_x} \quad (8)$$

where

|           |   |                              |
|-----------|---|------------------------------|
| $\beta_x$ | = | oocyte shape in stage x      |
| $l_x$     | = | long axis of oocyte stage x  |
| $s_x$     | = | short axis of oocyte stage x |



$$K_x = \left[ \frac{M_3}{M_1} \right]^{1/2} \quad (9)$$

where

$$M_1 = \frac{(D_{x1} + D_{x2} + D_{x3} + \dots + D_{xn})}{n}$$

$$M_{x3} = \left[ \frac{(D_{x1})^3 + (D_{x2})^3 + (D_{x3})^3 + \dots + (D_{xn})^3}{n} \right]^{1/3}$$

$K_x$  = oocyte diameter distribution of stage x

$n_x$  = number of oocyte counted

$D_x$  = mean diameter of stage x oocytes

$$D_x = \frac{(l_x + s_x)}{2} \quad (10)$$

$$N_{vx} = \frac{K_x}{\beta_x} \cdot \frac{N_{ax}^{3/2}}{V_{ix}^{1/2}} \quad (11)$$

where:

$N_{vx}$  = numbers of stage x oocytes per unit volume

$N_{ax}$  = numbers of stage x oocytes per unit area

$V_{ix}$  = Volume fraction occupied by the oocytes

stage x;  $= A_{ixp}$

In conclusion, the procedure for the stereological techniques can be divided into 2 phases. The first phase is to determine the total area fraction of stage x oocyte ( $A_{ax} = \Sigma A_{ox} / \Sigma A_{tx}$ ) using either the planimetric or the point counting methods. The second phase is to determine stage x oocyte numbers from the numerical density equation (equation 11).



## 4.2 OBJECTIVES

The objective of this study was to develop and compare:

- 1) The volume fractions of different stages of oocytes derived from three stereological methods [graph paper, mass and point counting] to assess maturity of *O. niloticus* ovaries.
- 2) The oocytes numbers derived from the numerical density equation [equation 11] with oocyte numbers estimated from the Gilson's fluid method.

## 4.3 METHODS

Six *O. niloticus* females at 22 week old, were studied in the present study. The fish were sacrificed and their total length and weight were recorded. The ovaries of the fish were carefully removed and the left ovaries were weighed individually and cut longitudinally. The ovaries were turned inside out and then placed in Gilson's fluid for ovarian digestion. The right ovaries were weighed and the middle section dissected and fixed in Bouin's fluid for stereological analysis.



#### 4.3.1 Stereological Methods

Histological sections were prepared according to the procedures described in section 2.2. Ten to fifteen sections were cut from each sample. The slides were stained with polychrome (section 2.2.1.2) and examined under the microscope and on a monitor (Figure 2.1). The oocyte stages were determined, according to criteria described in chapter 3 (Table 3.1).

Area fraction (volume fraction) of stage  $x$  oocytes ( $A_{ax}=V_{vx}$ ) on each slide was determined by each of the three stereological techniques: the two planimetric methods (graph paper and plastic sheet) and the point counting method (using the multipurpose graticule; M168).

##### 4.3.1.1. Graphical method (Graph paper method)

The stained ovarian sections were photographed using a Zeiss universal microscope. Twenty separate fields were randomly selected and photographed. The photographic slides were then projected onto graph paper and the outline of each oocyte stage was copied. The area occupied by each oocyte stage was counted and then computed as the proportional area occupied by that oocyte stage as shown in equation 12.



$$A_{axg} = \frac{A_{sx}}{A_{tsx}} \quad (12)$$

$A_{axg}$  = area fraction of stage x oocytes

$A_{sx}$  = area occupied by stage x oocytes in the photographic field

$A_{ts}$  = total ovary area of the photographic field

#### 4.3.1.2. Mass method (plastic weighing)

The stained sections were examined under the microscope and on the monitor as shown in Figure 2.1. Images of the ovarian sections appeared on the monitor to which a plastic sheet was attached. Twenty separate fields were selected randomly and the images were traced onto the plastic sheet. The outline of oocytes in different stages on the plastic sheets, were then cut and weighed. Area fractions of the oocytes in different stages were computed as shown in the equation 13.

$$A_{axm} = \frac{Wt_{sx}}{Wt_{ts}} \quad (13)$$

$A_{axm}$  = area fraction of oocyte stage x

$Wt_{sx}$  = weight of oocyte stage x in the field

$Wt_{ts}$  = total weight of the field (monitor)



#### 4.3.1.3. Intersection method (point counting)

The stained sections were examined under the microscope and on an external monitor via a video camera as shown in Figure 2.1. A multipurpose grid (M168, Weibel, 1979, Graticule Ltd, London) was placed in the photoeyepiece between the microscope and the video camera. The image of the grid was, therefore, overlaid on the ovarian section when viewed on the monitor (Figure 4.2). The number of grid points over lying each oocyte stages was counted and recorded. The area fraction was computed as shown in the equation below:

$$A_{axp} = \frac{A_{sx}}{A_{ts}} \quad (14)$$

$A_{axp}$  = area fraction of stage x oocytes

$A_{sx}$  = number of points occupied by stage x oocytes in the field

$A_{ts}$  = total number of point on the field (monitor)

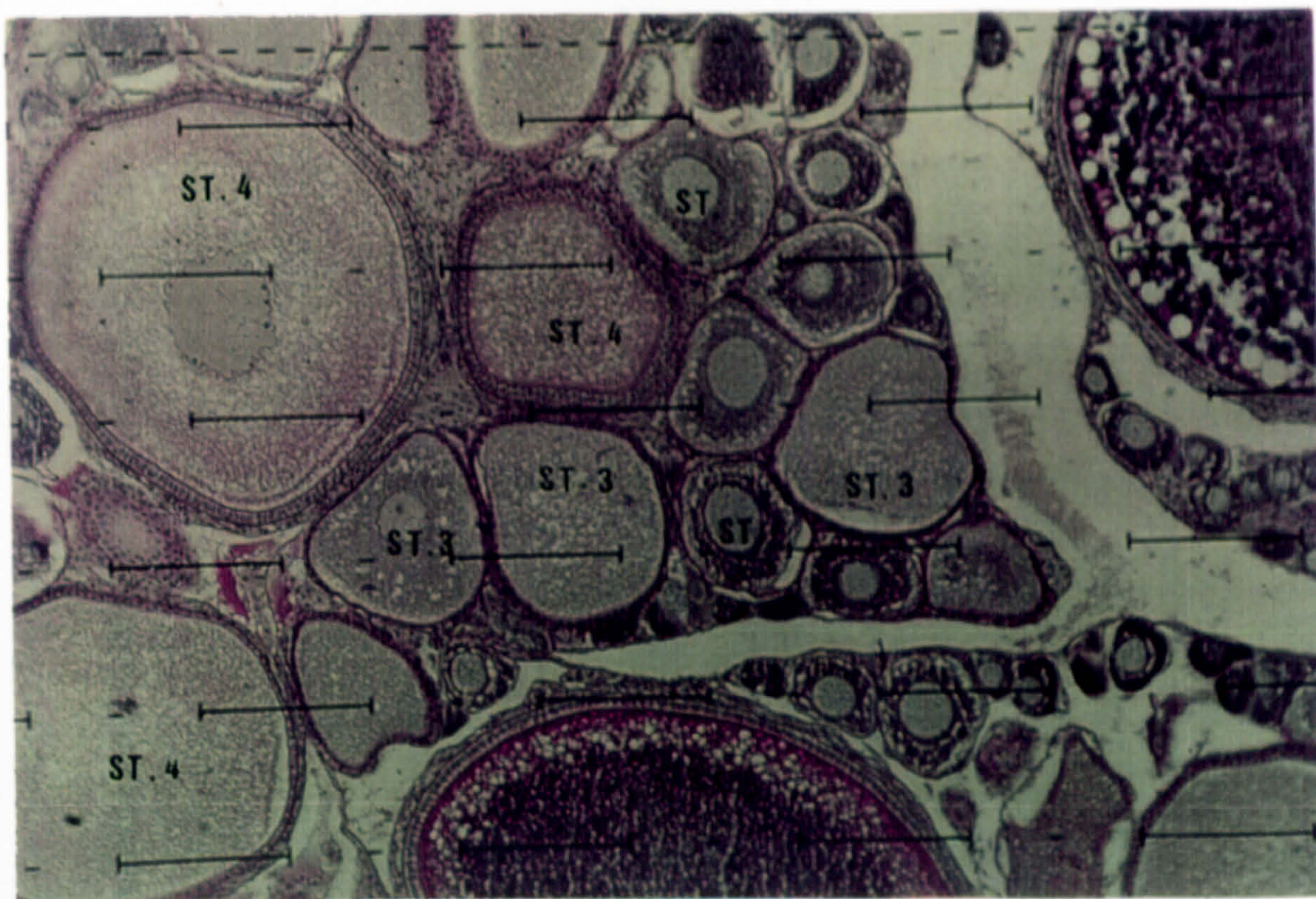
Only the area fractions for oocytes determined from the intersection method ( $A_{axp}$ ) were used to compute the number of oocytes per unit volume (equation 11).



Three additional parameters;  $N_{sx}$ ,  $K_x$  and  $\beta_x$  needed to be determined for the application of equation 11. The  $N_{sx}$  is the number of stage  $x$  oocytes in a unit area, and was computed from counting the number of stage  $x$  oocytes which lay in the total area of the field. Any oocyte which crossed the left and the top of the field on the monitor were included but any that fell across the right or the bottom borders were excluded as shown in Figure 4.2. The  $K_x$  was the diameter size distribution of stage  $x$  oocytes which was determined according to equation 9. The oocyte shape ( $\beta_x$ ) in stage  $x$  was determined from equation 8.

The total number of stage  $x$  oocytes in a fish was computed by multiplying the number of stage  $x$  oocytes in unit volume ( $N_{vx}$ ) with ovary volume, which was estimated from equation 15 in Figure 4.3.





$$\text{Total point } (A_{ts}) = 47 = 2.19 \text{ mm}^2/\text{monitor}$$

$$A_a \text{ stage 2} = 9/47 = 0.1915$$

$$A_a \text{ stage 3} = 5/47 = 0.1064$$

$$A_a \text{ stage 4} = 11/47 = 0.2340$$

$$A_a \text{ stage 5} = 5/47 = 0.1064$$

$$A_a \text{ stage 6} = 4/47 = 0.0851$$

$$N_a \text{ stage 2} = 38/2.19 \text{ mm}^2$$

$$N_a \text{ stage 3} = 4/2.19 \text{ mm}^2$$

$$N_a \text{ stage 4} = 2/2.19 \text{ mm}^2$$

Figure 4.2 Illustration demonstrates the estimation of area fractions of stage x oocytes ( $A_{ax}$ ) and numerical density ( $N_{ax}$ ) of different oocyte stages in a unit volume of *O. niloticus*.  $x150:1\text{cm}^2 = 0.0045\text{mm}^2$ .



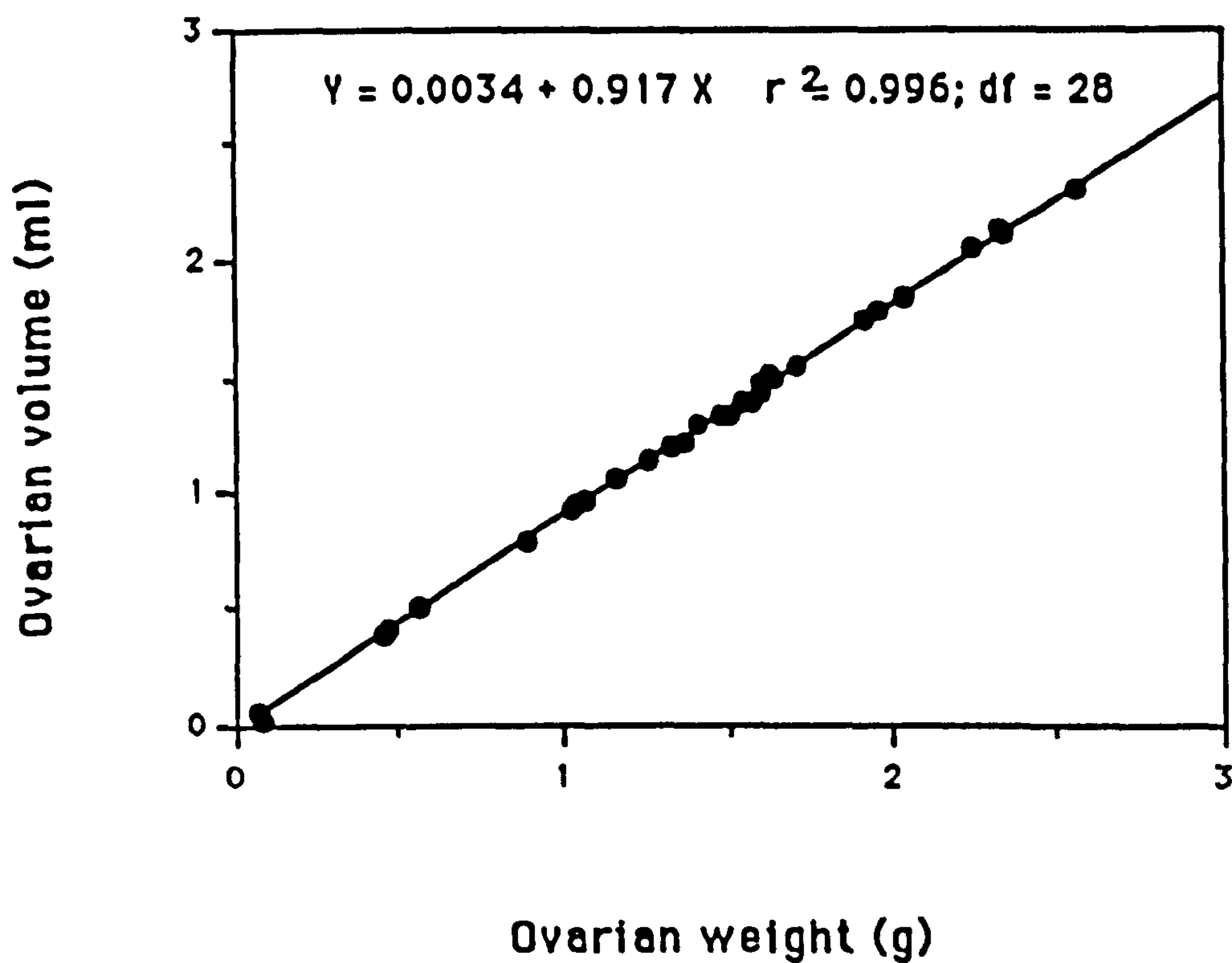


Figure 4.3 Regression between ovarian weight(X) and ovarian volume(Y) in *O.niloticus*.  $Y = 0.0034 + 0.917 X$  (equation 15);  $r^2 = 0.996$ ,  $P = 0.00$ ;  $df = 28$ .



#### 4.3.2 Gilson's Fluid Method

All six left ovaries were fixed in Gilson's fluid and occasionally agitated over a period of six weeks. Four ovaries from the total of six, were completely digested. The oocytes from these were separated from their surrounding tissues and counted.

The oocytes were washed in 3.6% formaldehyde buffer and filtered through three mesh sizes; a 1,500  $\mu\text{m}$ , 700  $\mu\text{m}$  and 300  $\mu\text{m}$  to produce four sized groups: 1) large, 2) medium, 3) small and 4) smallest oocytes. Each oocyte group was treated separately. The large oocytes were counted manually, their diameters recorded and then sorted into 75-100  $\mu\text{m}$  incremental classes (Table 4.7) according to their diameters. The last three oocyte groups were diluted at 1:10, 1:100 and 1:1000, respectively. Two subsamples were taken from each dilution. The diameters of each oocyte in these subsamples were recorded from microscopic observation, counted manually and subsequently grouped into the 75-100  $\mu\text{m}$  increment classes (Table 4.7).

The recorded oocyte diameters from each fish were then grouped into different oocyte stages according to the GSI of the fish, and 99% confidence limits of oocyte diameters of each stage given in Table 4.2a,b,c. Number of oocytes in different stages which were based on oocyte size were counted and the average numbers of oocytes in each stage



(e.g., stage x) in the subsample were then multiplied by dilution factors to obtain the stage x oocyte numbers in an ovary of the fish.

In order to determine the extent of overlap of oocyte stages in the discrete oocyte size classes, the oocytes from each size-class (Table 4.7) were then processed with resin embedding medium (section 2.2.1), cut and stained with polychrome as described in section 2.2.1.2. The oocyte stages within each size class were then determined according to chapter 3 (Table 3.1). The percentage of oocyte within each size class representing the different stages were then presented in Table 4.7.

The numbers of oocytes derived from numerical density equation (equation 11) were compared with the oocyte numbers derived from the Gilson's fluid.

#### 4.3.3 Statistical Analysis

Arcsine and log transformation were applied to the oocyte volume fractions and the oocyte numbers in different stages, respectively. The data were then subjected to analysis of variance using an unbalance GLM (General linear model: Minitab version 7.2 HP-UX). The level of significances was chosen to be 5%.



## 4.4 RESULTS

### 4.4.1 Age and Size of the Fish

There were no statistical differences ( $P>0.05$ ) in total length and weight between individual females used in this study. The median gonadosomatic index (GSIs) of the fish, however, were significantly different ( $P<0.05$ ; Table 4.1). This indicated that although the fish were the same age, their gonads had attained different levels of maturity. The ovaries were therefore divided into two groups according to their GSI; a) low GSI group (fish no's 1,2,3), and b) high GSI group (fish no's 4,5,6).

Table 4.1: Fish sizes and GSI of *O. niloticus* females used for the comparison of the three stereological methods and the Gilson's fluid method

| Fish no | Length(cm)        | Weight (g)        | GSI(%)            |
|---------|-------------------|-------------------|-------------------|
| 1.      | 13.7 <sup>a</sup> | 46.1 <sup>a</sup> | 0.85 <sup>a</sup> |
| 2.      | 14.6 <sup>a</sup> | 59.7 <sup>a</sup> | 0.28 <sup>a</sup> |
| 3.      | 12.5 <sup>a</sup> | 36.0 <sup>a</sup> | 0.29 <sup>a</sup> |
| 4.      | 13.5 <sup>a</sup> | 51.1 <sup>a</sup> | 3.90 <sup>b</sup> |
| 5.      | 13.5 <sup>a</sup> | 54.9 <sup>a</sup> | 3.12 <sup>b</sup> |
| 6.      | 13.1 <sup>a</sup> | 42.8 <sup>a</sup> | 2.99 <sup>b</sup> |
| Mean±SE | 13.1±0.29         | 42.8±3.50         | 2.58 ±1.15        |

The same superscripts within columns indicate no significant differences ( $P>0.05$ )



#### 4.4.2 Classification of Oocyte Diameters

Oocytes from the six ovaries examined in this study, were classified on their histo-morphological structures according to details in Table 3.1 (chapter 3). The diameter of different stages of oocytes were also separated into the low (Table 4.2a) and the high GSI groups (Table 4.2b). The diameters of the different developmental oocyte stages were measured and summarized into 5 oocyte stages (stages 2 to 6). In addition, to the observed oocyte diameters and means, the 99% confidence limits of the oocyte diameters in each stage are given in Table 4.2 a,b,c. These confidence limits were then used as the criteria to stage the oocytes from the high and low GSI groups.

Table 4.2a: Oocyte diameters of different developmental stages of the low *O. niloticus* GSI group

| Oocyte stages | Total number (n) | Oocyte diameters ( $\mu\text{m}$ ) |     |                  |                       |
|---------------|------------------|------------------------------------|-----|------------------|-----------------------|
|               |                  | Observed diameters                 |     |                  | 99% confidence limits |
|               |                  | min                                | max | mean $\pm$ SE    |                       |
| 2             | 2207             | 4                                  | 176 | 51.0 $\pm$ 0.62  | 10-100                |
| 3             | 95               | 110                                | 256 | 161.7 $\pm$ 2.59 | 140-200               |
| 4             | 103              | 172                                | 344 | 224.2 $\pm$ 3.98 | 180-300               |
| 5             | 49               | 214                                | 652 | 349.4 $\pm$ 16.6 | 301-450               |
| 6             | 91               | 422                                | 928 | 693.6 $\pm$ 15.2 | 451-950               |



Table 4.2b: Oocyte diameters of different developmental stages of the high *O. niloticus* GSI group

| Oocyte stages | Total number (n) | Oocyte diameters ( $\mu\text{m}$ ) |      |                  |                      |
|---------------|------------------|------------------------------------|------|------------------|----------------------|
|               |                  | Observed diameters                 |      |                  | 99%confidence limits |
|               |                  | min                                | max  | mean $\pm$ SE    |                      |
| 2             | 657              | 13                                 | 205  | 65.8 $\pm$ 1.23  | 21-160               |
| 3             | 56               | 182                                | 344  | 229.5 $\pm$ 5.9  | 161-300              |
| 4             | 27               | 308                                | 683  | 412.0 $\pm$ 17.1 | 301-500              |
| 5             | 15               | 527                                | 970  | 662.3 $\pm$ 28.3 | 501-700              |
| 6             | 103              | 501                                | 1965 | 1,566 $\pm$ 52.5 | 701-2000             |

Table 4.2c: Oocytes diameters of different developmental stages pooled from the low and high *O. niloticus* GSI group

| Oocyte stages | Total number (n) | Oocyte diameters ( $\mu\text{m}$ ) |      |                  |                      |
|---------------|------------------|------------------------------------|------|------------------|----------------------|
|               |                  | Observed diameters                 |      |                  | 99%confidence limits |
|               |                  | min                                | max  | mean $\pm$ SE    |                      |
| 2             | 2864             | 4                                  | 205  | 57.0 $\pm$ 0.58  | 20-120               |
| 3             | 151              | 110                                | 344  | 193.1 $\pm$ 3.98 | 140-220              |
| 4             | 130              | 172                                | 683  | 275.4 $\pm$ 8.56 | 221-300              |
| 5             | 64               | 214                                | 970  | 419.3 $\pm$ 19.3 | 301-700              |
| 6             | 194              | 422                                | 1965 | 993.3 $\pm$ 36.2 | 400-2000             |



#### 4.4.3 Comparison of Three Stereological Methods to Estimate Ovarian Volume Fractions of Oocytes

The estimated oocyte volume fractions derived from the mass, graphical and intersection methods, are shown in Tables 4.3a,b,c and Figure 4.4. These three methods yielded similar results ( $P>0.05$ ; Table 4.3d).

The volume fraction of stage 6 oocytes increased with the GSIs, while the volume fraction of stage 2 oocytes was inversely related to the GSIs (Figure 4.4; 4.5). There was a significant ( $P<0.05$ ) positive correlation between the volume fraction of stage 6 oocytes and the GSIs. This was best described by the following equation:

$$\% \text{ stage 6} = 43.22 + 50.1 \log \text{GSI} \quad r^2 = 0.84; P<0.05; df=86$$

where :

stage 6 = volume fraction of stage 6 oocytes (%)  
GSI = gonadosomatic index (%)

Although all the three stereological methods could be used to estimate the volume fractions of oocytes, the advantages and disadvantages of each method was considered in order to select an appropriate method to use in further studies.



Table 4.3a: Volume fractions of oocytes in different stages from individual *O. niloticus* estimated by the mass method

| Fish no | Ovarian volume fraction (% mean $\pm$ SE) |                |                 |                 |                 |
|---------|---|----------------|-----------------|-----------------|-----------------|
|         | stage 2                                   | stage 3        | stage 4         | stage 5         | stage 6         |
| 1       | 9.0 $\pm$ 0.05                            | 4.0 $\pm$ 0.05 | 4.2 $\pm$ 0.06  | 10.6 $\pm$ 0.17 | 60.5 $\pm$ 0.25 |
| 2       | 27.3 $\pm$ 0.19                           | 8.4 $\pm$ 0.80 | 8.6 $\pm$ 0.15  | 24.9 $\pm$ 0.22 | 9.7 $\pm$ 0.20  |
| 3       | 29.2 $\pm$ 0.14                           | 9.3 $\pm$ 0.12 | 14.1 $\pm$ 0.22 | 9.3 $\pm$ 0.25  | 14.1 $\pm$ 0.18 |
| 4       | 5.4 $\pm$ 0.07                            | 3.7 $\pm$ 0.05 | 4.2 $\pm$ 0.10  | 4.2 $\pm$ 0.12  | 63.3 $\pm$ 0.27 |
| 5       | 3.3 $\pm$ 0.03                            | 1.9 $\pm$ 0.03 | 2.8 $\pm$ 0.04  | 2.9 $\pm$ 0.06  | 85.0 $\pm$ 0.18 |
| 6       | 1.5 $\pm$ 0.02                            | 0.8 $\pm$ 0.02 | 1.0 $\pm$ 0.18  | 7.2 $\pm$ 0.11  | 80.8 $\pm$ 0.14 |

Fish no's 1, 2, 3 from low GSI group.

Fish no's 4, 5, 6 from high GSI group.

Table 4.3b: Volume fractions of oocytes in different stages from individual *O. niloticus* estimated by the graphical method

| Fish no | Ovarian volume fractions (% mean $\pm$ SE) |                 |                 |                 |                 |
|---------|--|-----------------|-----------------|-----------------|-----------------|
|         | stage 2                                    | stage 3         | stage 4         | stage 5         | stage 6         |
| 1       | 6.3 $\pm$ 0.45                             | 4.0 $\pm$ 0.38  | 4.6 $\pm$ 0.23  | 7.2 $\pm$ 0.49  | 56.2 $\pm$ 1.13 |
| 2       | 18.4 $\pm$ 0.82                            | 5.1 $\pm$ 0.69  | 10.5 $\pm$ 0.55 | 32.4 $\pm$ 0.34 | 8.0 $\pm$ 0.42  |
| 3       | 29.8 $\pm$ 0.38                            | 12.5 $\pm$ 0.24 | 22.4 $\pm$ 0.14 | 8.6 $\pm$ 0.29  | 13.4 $\pm$ 0.86 |
| 4       | 6.6 $\pm$ 0.39                             | 3.0 $\pm$ 0.31  | 3.0 $\pm$ 0.18  | 6.6 $\pm$ 0.16  | 65.9 $\pm$ 0.55 |
| 5       | 2.8 $\pm$ 0.17                             | 0.9 $\pm$ 0.52  | 2.7 $\pm$ 0.36  | 2.5 $\pm$ 0.12  | 86.3 $\pm$ 0.87 |
| 6       | 1.6 $\pm$ 0.15                             | 0.1 $\pm$ 0.11  | 0.2 $\pm$ 0.21  | 5.3 $\pm$ 0.05  | 85.2 $\pm$ 0.72 |

Fish no's 1, 2, 3 from low GSI group.

Fish no's 4, 5, 6 from high GSI group.



Table 4.3c: Volume fractions of oocytes in different stages from individual *O. niloticus* estimated by the intersection method

| Fish no | Ovarian volume fraction (% mean $\pm$ SE) |                |                 |                 |                 |
|---------|---|----------------|-----------------|-----------------|-----------------|
|         | stage 2                                   | stage 3        | stage 4         | stage 5         | stage 6         |
| 1       | 10.7 $\pm$ 0.11                           | 4.7 $\pm$ 0.34 | 4.0 $\pm$ 0.41  | 5.1 $\pm$ 0.22  | 59.9 $\pm$ 0.20 |
| 2       | 24.3 $\pm$ 0.12                           | 4.0 $\pm$ 1.30 | 6.8 $\pm$ 0.63  | 25.6 $\pm$ 0.28 | 6.1 $\pm$ 0.26  |
| 3       | 23.8 $\pm$ 0.46                           | 7.8 $\pm$ 1.09 | 10.8 $\pm$ 0.81 | 14.4 $\pm$ 0.42 | 12.1 $\pm$ 0.15 |
| 4       | 9.9 $\pm$ 0.98                            | 5.1 $\pm$ 1.04 | 3.6 $\pm$ 0.91  | 2.9 $\pm$ 0.39  | 70.0 $\pm$ 1.73 |
| 5       | 4.4 $\pm$ 0.17                            | 2.3 $\pm$ 0.17 | 2.9 $\pm$ 1.05  | 2.6 $\pm$ 0.52  | 78.5 $\pm$ 1.73 |
| 6       | 2.7 $\pm$ 0.65                            | 1.6 $\pm$ 0.47 | 1.4 $\pm$ 2.69  | 3.9 $\pm$ 2.90  | 82.6 $\pm$ 0.82 |

Fish no's 1, 2, 3 from low GSI group.

Fish no's 4, 5, 6 from high GSI group.

Table 4.3d: Analysis of variance of the three stereological methods used to estimate ovarian volume fractions of *O. niloticus* oocytes

| Source      | df   | SS       | MS      | F      | P        |
|-------------|------|----------|---------|--------|----------|
| Methods (M) | 2    | 597.9    | 299.0   | 2.28   | 0.103 NS |
| Fish (F)    | 5    | 3037.2   | 670.4   | 4.62   | 0.000    |
| Stages (S)  | 4    | 225132.7 | 56283.2 | 428.52 | 0.000    |
| MxF         | 10   | 1652.8   | 165.3   | 1.26   | 0.25 NS  |
| MXS         | 8    | 23785.0  | 2973.1  | 22.64  | 0.000    |
| FxS         | 20   | 250998.1 | 12549.9 | 95.55  | 0.000    |
| MXFXS       | 40   | 20062.0  | 501.5   | 3.82   | 0.000    |
| Error       | 1715 | 225252.1 | 131.3   |        |          |



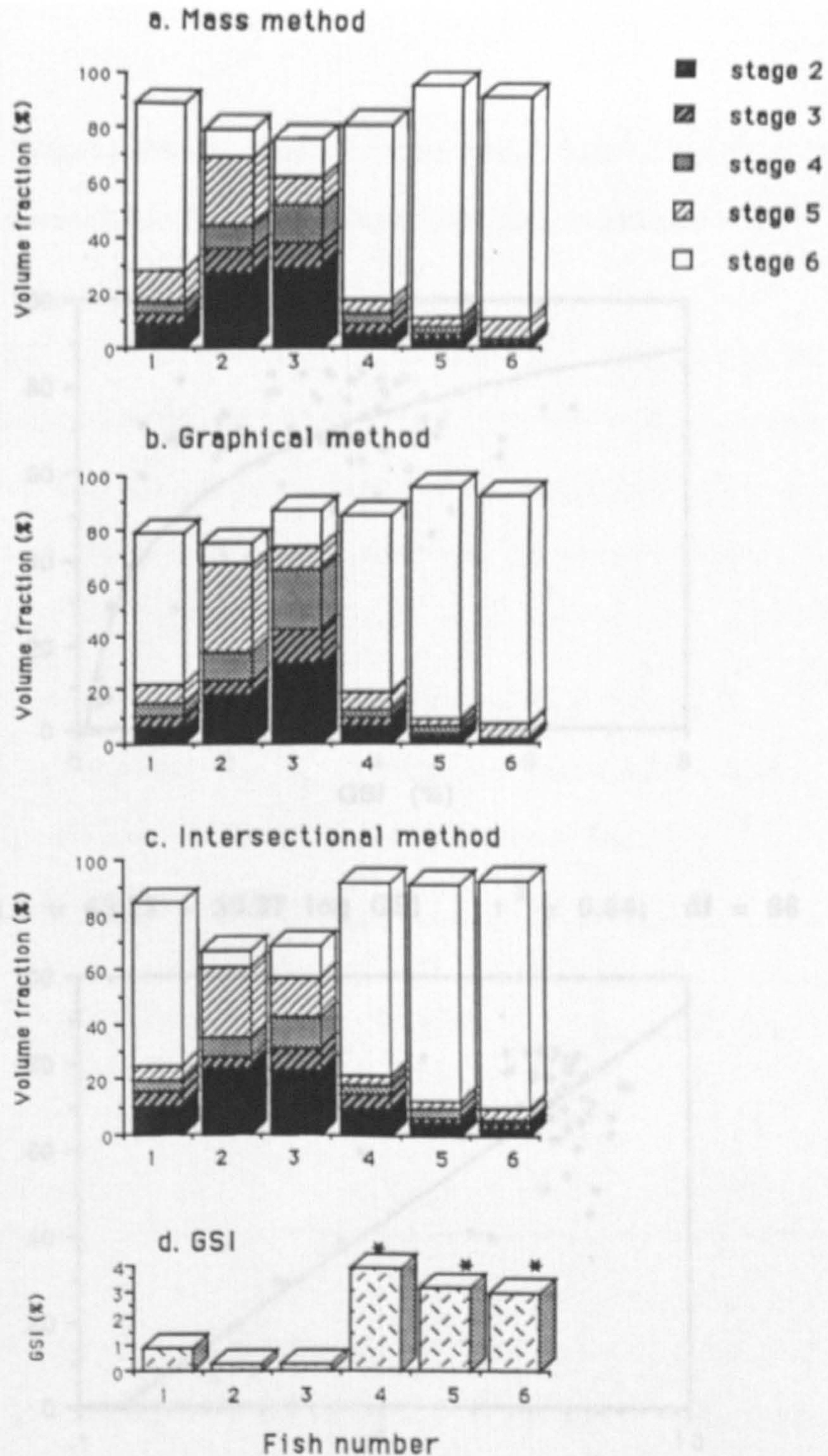
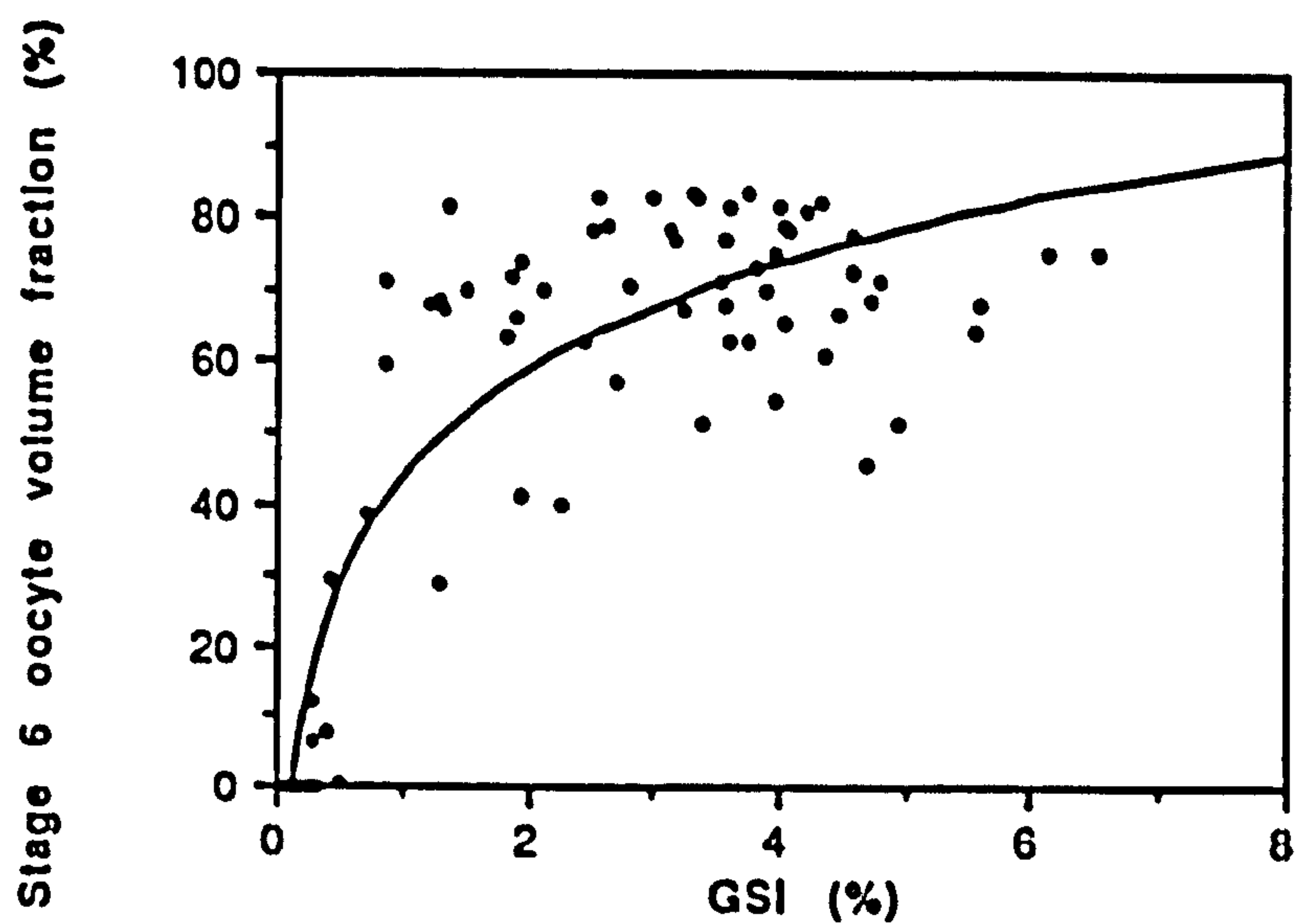


Figure 4.4: Volume fractions of *O.niloticus* oocytes estimated by three stereological techniques: a) mass method; b) graphical method; c) intersection method; compared with d) GSI. Fish no's 1, 2, 3 were from the low GSI group and fish no's 4, 5, 6 were from the high GSI group. (\*) indicates significant ( $P < 0.05$ ) between the low and high GSI fish.



(a)



(b)

$$\text{St.6} = 43.22 + 50.07 \log \text{GSI} \quad r^2 = 0.84; \quad \text{df} = 86$$

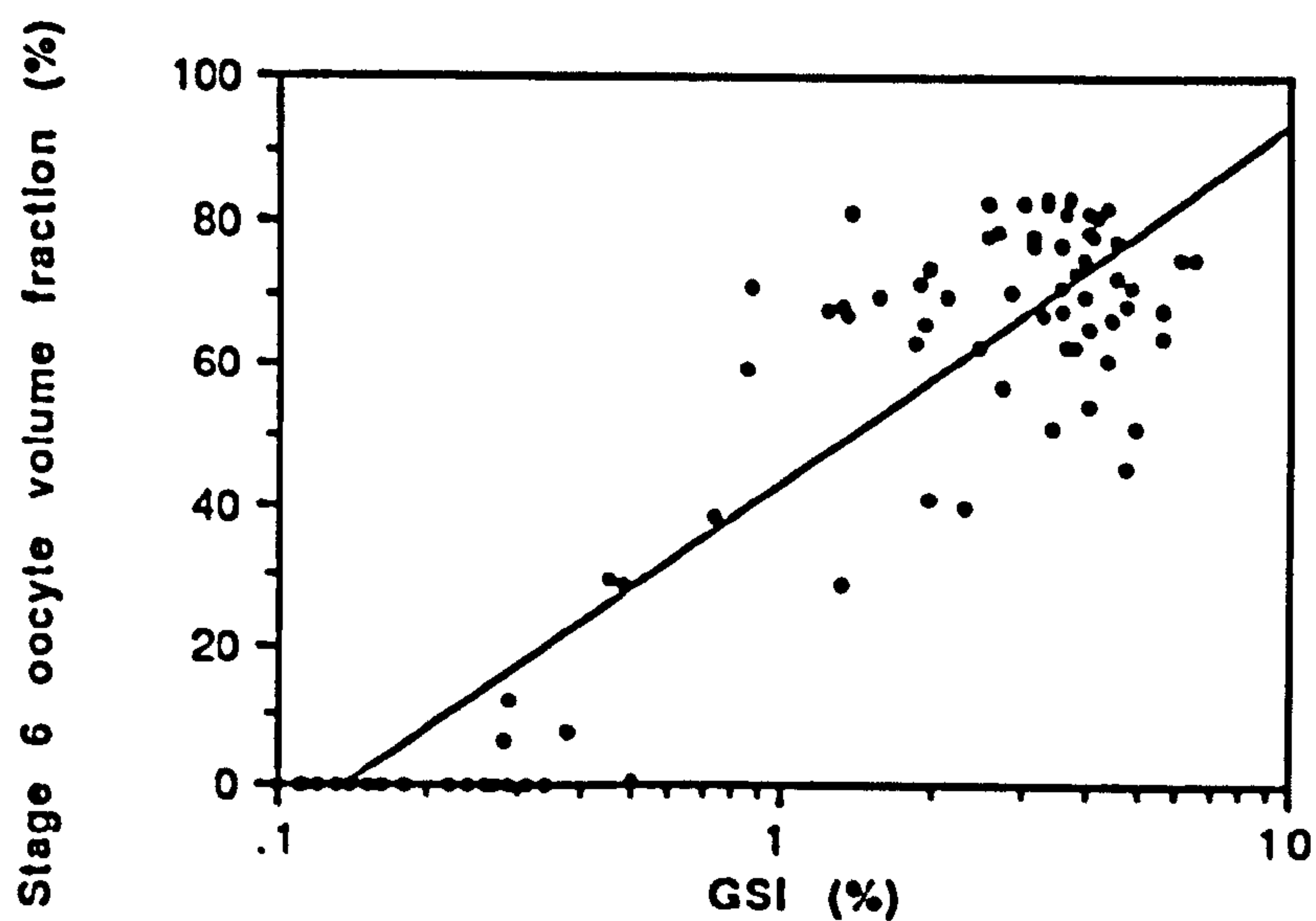


Figure 4.5: (a) Relationship between stage 6 oocyte volume fractions and GSI of *O. niloticus*. (b) Relationship of stage 6 oocyte volume fraction on semilog scale of GSI. % Stage 6 volume fractions =  $43.2 + 50.1 \log \text{GSI}$   $r^2 = 0.84$ ;  $P < 0.05$ ;  $\text{df} = 86$ .



#### 4.4.4 Time Requirement for Estimating the Ovarian Volume Fractions Using the Three Stereological Methods

The time required to estimate the volume fractions of each stereological methods was recorded (Table 4.4). Drawing of the mass and the graphical methods, required the average time of 3.3 and 4.0 mins per sample, respectively.

Table 4.4: Time taken for each step for three stereological methods to determine the volume fractions of oocytes in different stages

| Methods         | Time (mins) |     |       |       |       |
|-----------------|-------------|-----|-------|-------|-------|
|                 | Draw        | Cut | Weigh | Count | Total |
| 1. Mass         | 3.3         | 7.1 | 1.7   | -     | 12.1  |
| 2. Graphic      | 4.0         | -   | -     | 7.1   | 11.1  |
| 3. Intersection | -           | -   | -     | 2.6   | 2.6   |

For the mass method, the sheets had to be cut and weighed, while the graphical method only required counting the number of squares occupied by the oocytes to obtain the volume fraction. Thus, the total time per sample required for these two methods was 12.1 and 11.1 mins, respectively. The intersection method, in which the grid points were applied (Weibel, 1979), required the shortest time. In this study, the intersection method required only 2.6 mins for each sample, which was 4.2 to 4.5 times shorter than the graphic and the mass methods, respectively.



The intersection method was therefore the most appropriate and efficient method for estimating the volume fraction of the different oocyte stages in *O.niloticus*. This method was, therefore, selected for the computation of oocyte numbers (equation 11).

#### 4.4.5 Diameter Distribution Coefficient (K) and Shape Coefficient ( $\beta$ ) of Oocytes in Different Developmental Stages

Weibel (1979) had shown that when the size of particles is normally distributed and the coefficient of variation is less than 25%, then the K value will fall between 1.00-1.07. If the particle sizes are not normally distributed and/or show a coefficient of variation greater than 25% then K will be higher than 1.07.

The diameter distribution (K; Table 4.5) of the oocyte in stages 3, 4 and 6 from the low GSI group and the oocyte stages 3, 4 and 5 of the high GSI group were normally distributed, while the others were not (Figure 4.6). The data was therefore, log transformed prior to statistical analysis.

In this study, the shape coefficients ( $\beta$ ) of the oocytes in different stages fell into the narrow range of 1.08-1.10, suggesting that the shape of the oocytes in different stages was either elliptical or oval in shape (Table 4.5).



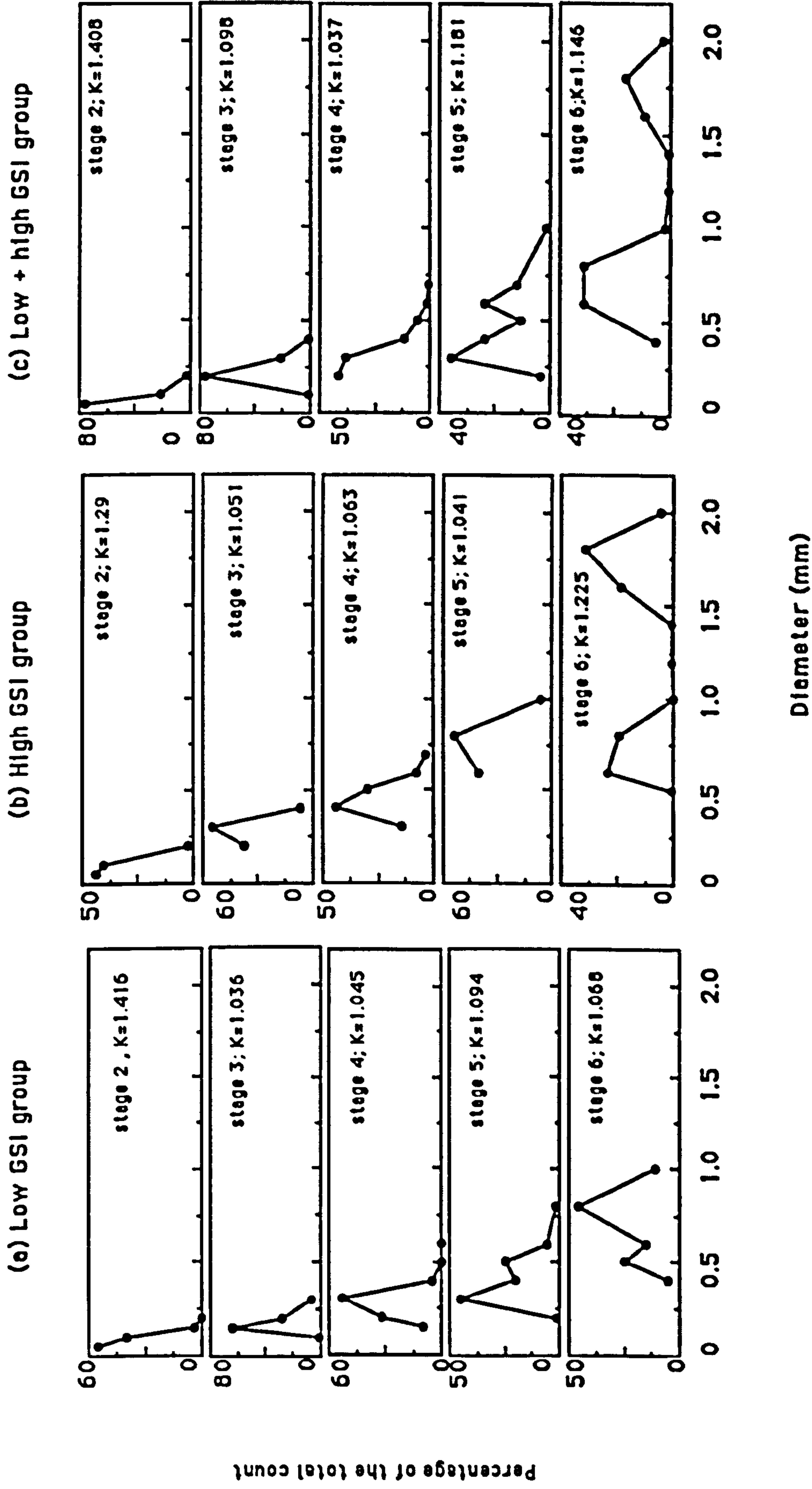


Figure 4.6: Oocyte diameter distribution (K) of different *O. niloticus* oocyte developmental stages. (a) Low GSI group; (b) High GSI group; (c) Pooled from the low + high GSI groups



Table 4.5: Diameter size distributions (K) and shape coefficients ( $\beta$ ) of *O. niloticus* oocytes in different developmental stages and GSI

| Stages | GSI groups |         |       |         |            |         |
|--------|------------|---------|-------|---------|------------|---------|
|        | Low        |         | High  |         | Low + High |         |
|        | K          | $\beta$ | K     | $\beta$ | K          | $\beta$ |
| 2      | 1.416      | 1.096   | 1.290 | 1.102   | 1.408      | 1.098   |
| 3      | 1.036      | 1.094   | 1.051 | 1.090   | 1.098      | 1.093   |
| 4      | 1.045      | 1.083   | 1.063 | 1.090   | 1.037      | 1.087   |
| 5      | 1.094      | 1.088   | 1.041 | 1.087   | 1.181      | 1.088   |
| 6      | 1.068      | 1.096   | 1.225 | 1.097   | 1.146      | 1.098   |

#### 4.4.6 Comparison of Oocyte Numbers Estimated by Stereology and the Gilson's Fluid Method

The stage 2 oocytes were the most dominant stage in every fish (Table 4.6a,b). The numbers of the different oocyte stages from ovaries of the same fish estimated by the numerical density equation and the Gilson's fluid method were not significantly different ( $P>0.05$ ; Table 4.6c).



Table 4.6a: Numbers of oocytes estimated by the  
numerical density stereological method

| Fish<br>No | Oocyte numbers (mean $\pm$ SE) |                     |                    |                   |                 |
|------------|--------------------------------|---------------------|--------------------|-------------------|-----------------|
|            | stage 2                        | stage 3             | stage 4            | stage 5           | stage 6         |
| 1          | 136488<br>$\pm$ 23031          | 3835<br>$\pm$ 768   | 2523<br>$\pm$ 915  | 234<br>$\pm$ 49   | 434<br>$\pm$ 46 |
| 2          | 91242<br>$\pm$ 14912           | 1188<br>$\pm$ 290   | 330<br>$\pm$ 140   | 161<br>$\pm$ 63   | 31<br>$\pm$ 6   |
| 3          | 879462<br>$\pm$ 103397         | 13324<br>$\pm$ 2451 | 6333<br>$\pm$ 1820 | 4177<br>$\pm$ 945 | 5<br>$\pm$ 3    |
| 4          | 203155<br>$\pm$ 21926          | 13151<br>$\pm$ 2329 | 843<br>$\pm$ 203   | 256<br>$\pm$ 87   | 341<br>$\pm$ 33 |
| 5          | 277517<br>$\pm$ 38203          | 24609<br>$\pm$ 3839 | 635<br>$\pm$ 237   | 217<br>$\pm$ 116  | 322<br>$\pm$ 39 |
| 6          | 65715<br>$\pm$ 14547           | 4447<br>$\pm$ 1065  | 152<br>$\pm$ 60    | 143<br>$\pm$ 65   | 171<br>$\pm$ 18 |

Table 4.6b: Oocyte numbers estimated by the  
Gilson's fluid method

| Fish<br>No | Oocyte numbers (mean $\pm$ SE) |                    |                    |               |              |
|------------|--------------------------------|--------------------|--------------------|---------------|--------------|
|            | stage 2                        | stage 3            | stage 4            | stage 5       | stage 6      |
| 1          | 92227<br>$\pm$ 16466           | 4463<br>$\pm$ 1737 | 2729<br>$\pm$ 1000 | 399 $\pm$ 180 | 215 $\pm$ 52 |
| 4          | 128299<br>$\pm$ 57480          | 9884<br>$\pm$ 3472 | 3387<br>$\pm$ 2444 | 574 $\pm$ 388 | 178 $\pm$ 93 |
| 5          | 291968<br>$\pm$ 83462          | 6932<br>$\pm$ 245  | 592<br>$\pm$ 93    | 166 $\pm$ 2   | 226 $\pm$ 83 |
| 6          | 53327<br>$\pm$ 35128           | 2325<br>$\pm$ 1474 | 823<br>$\pm$ 431   | 774 $\pm$ 826 | 171 $\pm$ 56 |



Table 4.6c: Analysis of variance table comparing  
oocyte numbers estimated by the  
stereological method and the Gilson's fluid  
method

| Source         | Df  | SS                   | MS                   | F     | P        |
|----------------|-----|----------------------|----------------------|-------|----------|
| Methods<br>(M) | 1   | $17.12 \cdot 10^8$   | $17.12 \cdot 10^8$   | 0.62  | 0.432 NS |
| Fish (F)       | 3   | $4.50 \cdot 10^{10}$ | $1.50 \cdot 10^{10}$ | 5.43  | 0.001    |
| Stages<br>(S)  | 4   | $5.48 \cdot 10^{11}$ | $1.37 \cdot 10^{11}$ | 49.56 | 0.000    |
| MxF            | 3   | $1.13 \cdot 10^9$    | $0.38 \cdot 10^9$    | 0.14  | 0.938 NS |
| MxS            | 4   | $4.86 \cdot 10^9$    | $1.21 \cdot 10^9$    | 0.44  | 0.780 NS |
| FxS            | 12  | $1.58 \cdot 10^{11}$ | $1.32 \cdot 10^{10}$ | 4.76  | 0.000    |
| MxExS          | 12  | $7.37 \cdot 10^9$    | $0.61 \cdot 10^9$    | 0.22  | 0.990 NS |
| Error          | 400 | $1.11 \cdot 10^{12}$ | $0.28 \cdot 10^{10}$ |       |          |



Table 4.7: Percentage of oocytes in different developmental stages in each oocyte diameter size class which corresponded to stage 6 oocytes in Table 4.2c. The oocyte classification in this table was based on their histo-morphological structures after the oocytes were processed in Gilson's fluid. The samples were pooled from low and high GSI group.

| Oocyte<br>diameters<br>( $\mu\text{m}$ ) | Percentage overlap of oocytes in different<br>developmental stages |         |         |         |         |
|--|--|---------|---------|---------|---------|
|  | stage 2  | stage 3 | stage 4 | stage 5 | stage 6 |
| 400-500                                  | -  | -       | 59.5    | 40.0    | 0.5     |
| 501-600                                  | -  | -       | 7.5     | 90.1    | 2.3     |
| 601-700                                  | -  | -       | -       | 72.2    | 27.8    |
| 750-850                                  | -  | -       | -       | 27.9    | 72.2    |
| 801-900                                  | -  | -       | -       | 5.4     | 94.6    |
| 975-1050                                 | -  | -       | -       | 1.6     | 98.4    |
| 1051-1150                                | -  | -       | -       | 1       | 99.0    |
| 1151-1250                                | -  | -       | -       | -       | 100     |
| 1400-1500                                | -  | -       | -       | -       | 100     |
| 1501-1600                                | -  | -       | -       | -       | 100     |
| 1601-1700                                | -  | -       | -       | -       | 100     |
| 1701-1800                                | -  | -       | -       | -       | 100     |
| 1801-1900                                | -  | -       | -       | -       | 100     |
| 1901-2000                                | -  | -       | -       | -       | 100     |
| 2001-2100                                | -  | -       | -       | -       | 100     |

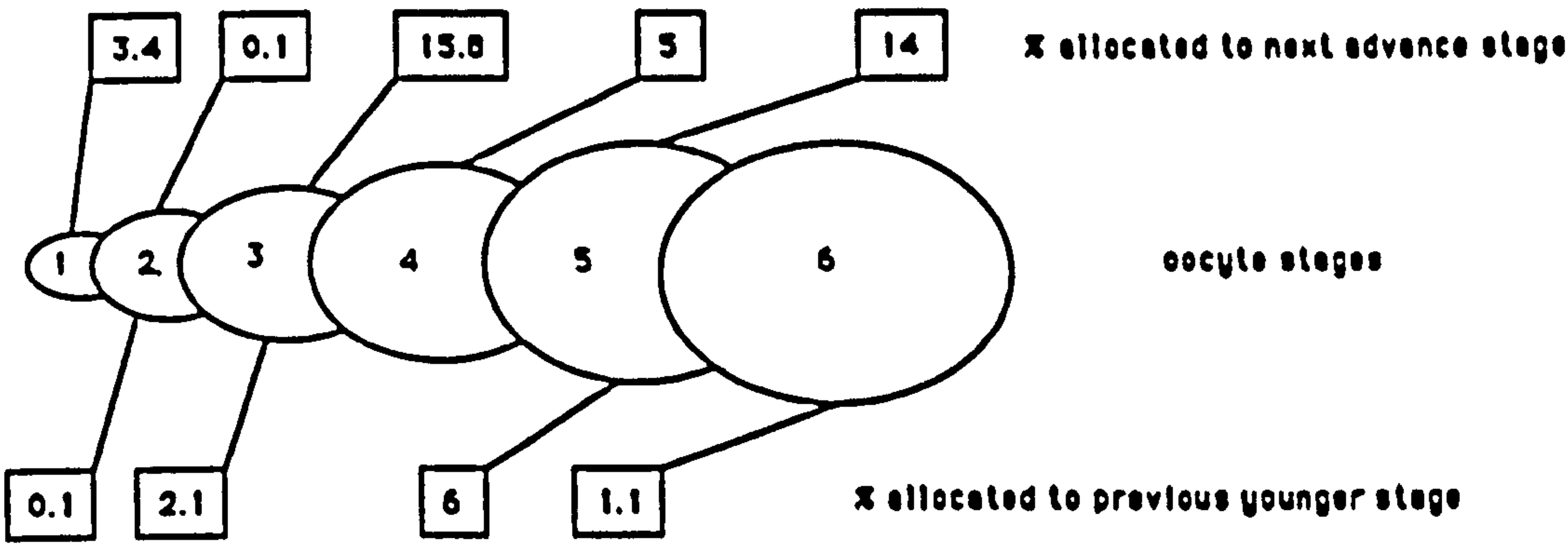


Figure 4.7: The degree of overlap of oocytes when based on oocyte diameters. The 99% confidence limits of absolute oocyte diameters determined from histological techniques, were used as the criteria for staging oocytes obtained by the Gilson's fluid method. The % of oocytes allocated to the advanced or younger oocyte stages are also shown. For example, in Table 4.2a, the absolute minimum and maximum oocyte diameters of stages 2 were 4 and 176  $\mu\text{m}$ , respectively, and the upper and lower 99% confidence limits criteria used to classify stage 2 oocytes were 10 - 100 $\mu\text{m}$ . Therefore, the % of stage 2 oocytes which ranged 4 - 9 $\mu\text{m}$  and 101 - 176 $\mu\text{m}$  equal 0.1% allocated to stage 1 and stage 3 oocytes (Figure 4.8a). Such data are given for fish with different GSI:

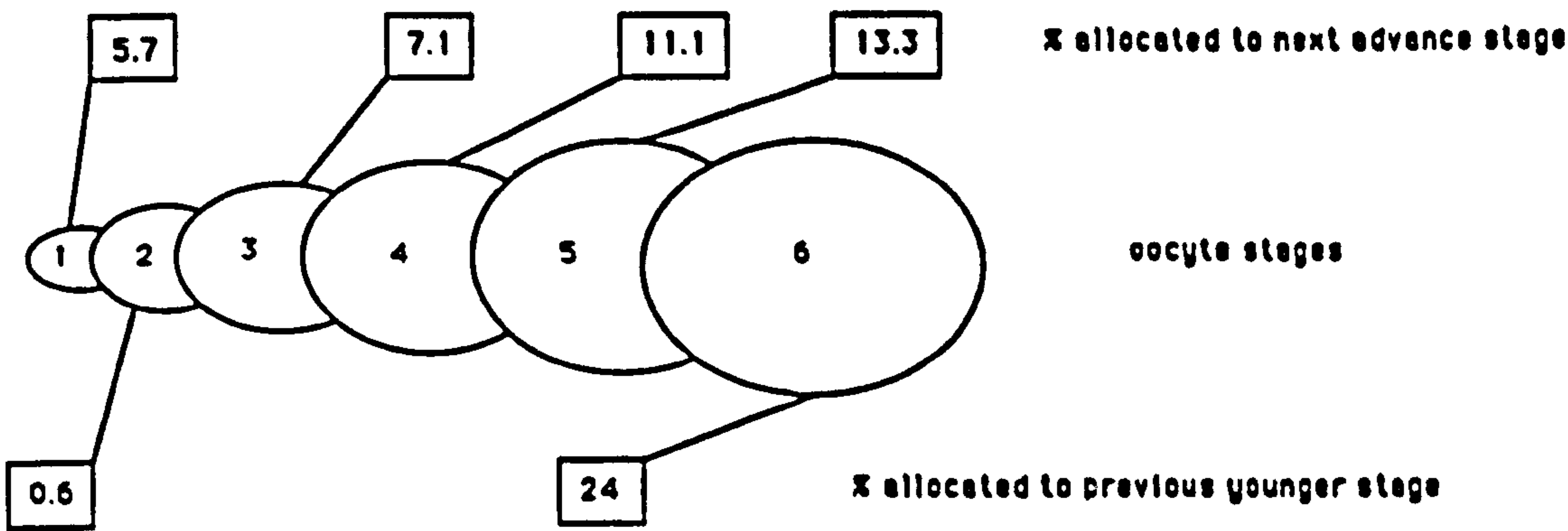
- a) Data from low GSI group
- b) Data from high GSI group
- c) Pooled data from the low and high GSI groups



(a) Low GSI group



(b) High GSI group



(c) Low + high GSI group

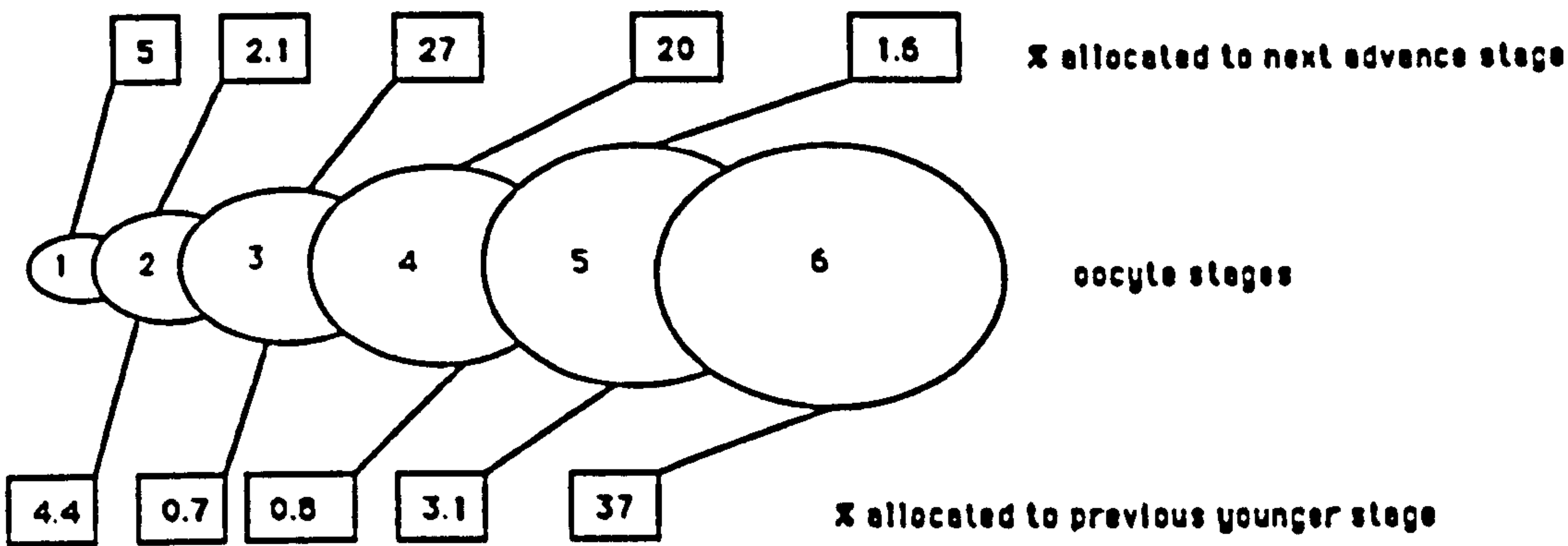


Figure 4.7



#### 4.5 DISCUSSION

The present study showed that the oocyte numbers calculated from the numerical density equation of the stereological method were similar to that of the Gilson's fluid method. The results in the present study correspond to those of Emerson *et al.*, (1990), even though they used a different method (automatic counting) to count oocyte numbers obtained by the Gilson's fluid method. Therefore, either the numerical density or the Gilson's method could be used to estimate oocyte numbers.

The Gilson's fluid method, however, has many disadvantages. The fluid is toxic and the digestion process is time consuming when compared to the stereological method. The stereological method, in addition, classifies the oocyte stages from histological sections. Thus, the later is more accurate as it is based on the oocyte structures rather than oocyte diameters (West, 1990).

The present study showed that the estimate of oocyte numbers in various oocyte stages can be influenced by the criteria used to classify oocytes. The stereological method classifies oocytes into different developmental stages based on the histo-morphological structure of each oocyte stage. Thus, this method should be more accurate for staging oocytes. In the Gilson's fluid method, however, the oocyte stages were based on the 99% confidence limits of



the actual oocyte diameters of the different stages (Table 4.2a,b,c). Although these size classes of the different "oocyte stages" were discrete, there was a degree of overlap when their actual and observed diameters are considered (Figure 4.7). The degree of overlap was also influenced by the state of gonadal development (low/high GSI). When the respective oocyte stages from the low and high GSI groups were combined the overlap ranged between 0.7 to 37%. The separation of the respective ovaries into low and high GSI groups reduced this overlap to between 0.1 to 24%.

In addition, the application of the Gilson's technique showed that the oocyte size classes between 400 to 1150 $\mu$ m in Table 4.7 (which correspond to stage 6 oocytes in Table 4.2c) contained 7.5 - 59.5% of stage 4, 1 - 90% of stage 5 and 1 - 99% of stage 6 oocytes. This result highlights the confounding effect of using oocyte diameter as the criteria for staging oocytes in the Gilson's fluid method. Although these criteria were based on the 99% confidence limits of the actual oocyte diameters, the error of this method is considerably high (Table 4.7).

There was no statistical difference ( $P>0.05$ ), however, between the oocyte numbers estimated from the Gilson's fluid and stereological methods which indicates that the numbers of oocytes estimated from stereological technique also contains some errors. These errors may be caused by



*Placopecten magellanicus*, the volume fractions of ripe gametes increased higher than in developing gametes during breeding season. Therefore, the volume fraction of stage 6 or ripe oocytes may also be used to determine the maturity of the fish. The volume fraction estimated by the intersection method was therefore chosen to estimate maturity of *O. niloticus* throughout this study.



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## CHAPTER 5

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## 5. SEXUAL DIFFERENTIATION, OVARIAN DEVELOPMENT AND HORMONAL PROFILES OF *O. niloticus* DURING PUBERTY

### 5.1 INTRODUCTION

Genetic and environmental factors influence the age and size of sexual differentiation and puberty of teleost species. These two stages are important periods in aquaculture. For example, correct timing of sexual differentiation is critical for the production of all male tilapias by sex reversal techniques using exogenous steroid hormones. The timing of maturation is required to provide more precise estimation of spawning period and to improve the dynamics of age-size composition of stocks to obtain the maximum fish production.

#### 5.1.1 Sexual Differentiation

External stimulation such as temperature, has been reported to affect the sexual differentiation of poikilothermic vertebrates (Bull and Vogt, 1979). In turtles (Bull and Vogt, 1979; Bull, Vogt and Bulmer, 1982a; Bull, Vogt and McCoy, 1982b) and atherinid fish, *Menidia menidia* (Conover and Kynard, 1981; Conover, 1984) low or high temperature during the phase of embryonic development can lead to a high proportion of either male or female offsprings, respectively.



Male or female sex ratios of teleosts such as tilapias, have been investigated and can be manipulated by using exogenous steroid hormones prior to onset of sexual differentiation (Yamamoto, 1969). The onset of sexual differentiation of tilapias depends on fish species and rearing temperature. For example, in *O. mossambicus* fry reared at 20°C, sex differentiation occurred by 20 day post-hatch (Nakamura and Takahashi, 1973), whereas the sex differentiation in *O. niloticus* fry reared at 25-26°C occurred at 30 to 33 day post-hatch (Alvencia-Casauay and Carino, 1988).

Under farm conditions where the age of the fry may not be known, the critical fry size of "less than 12 mm" has been advocated for the initial administration of steroid hormones (Tayamen and Shelton, 1978; Nakamura and Iwahashi, 1982; Hopkins, Shelton and Engle, 1979). The size of the fry, however, will depend on culture conditions such as stocking density, food, etc. Fry of less than 12 mm may, therefore, consist of mixed ages structure in which the older but smaller fry may have completed their sexual differentiation. There is no information on the effect of such factors on the onset of sexual differentiation. A trial was, therefore, conducted to study the effect of stocking densities on the onset of sexual differentiation of *O. niloticus*.



### 5.1.2 Gonadal Development and Gonadosomatic Index

On completion of sexual differentiation, the male gonad develops into testis whereas the female gonad forms into an ovary. Each gonad generally consists of two lobes, which joins posteriorly and enters the genital papilla.

The size of the ovary in fish increases with ovarian development, fish size and age. Thus, the ratio of gonad weight to body weight, or the gonadosomatic index (GSIs), is a widely used indicator of gonadal activity and maturity in fishes. The maximum GSIs do vary with the reproductive strategy of the fish species. In semelparous (e.g. salmon) and iteroparous fish (e.g. rainbow trout), the GSIs can reach 30% and 15% (Billard, et al.,1978), respectively, whereas in the multiple spawners like tilapias the maximum GSIs typically range between to 3.5% and 7% (Babiker and Ibrahim,1979a; Dadzie and Wangila, 1980).

It is common to use the GSIs in reproductive studies for comparing different treatment groups or for comparing seasonal maturity differences (de Vlaming, et al.,1982). In rainbow trout for example, GSIs are small at about 0.1% to 5% during summer (June) and gradually increase to 13% to 25% during winter (November to March) (Lambert, Bosman, Van den Hurk, Van Oordt, 1978; Sumpter and Scott,1989). Similarly the GSIs of goldfish is low (2% to 4%) during winter months (August to February) and rapidly increase



(6.4%) in spring (April) and peaks at 11% in early summer (May and June) (Kobayashi, Aida and Hanyu, 1986). In wild tilapias the overall mean GSIs are lower. For example in *O. niloticus*, the mean female GSIs are less than 1% in January and increase to 1 - 2% in April (Latif and Rashid, 1972). These GSIs of these wild species ranged between 0.6% to 1.18% in the ripening ovary, 1.17% to 3.58% in the ripe ovary and 0.07% to 0.31% in the spent ovary (Babiker and Ibrahim, 1979a).

#### 5.1.3 Maturity and Spawning in Response to External Stimuli

Generally tilapias will spawn when the temperature is favourable. For example, in the genus *Oreochromis*, natural reproduction occurs at temperatures above 22°C and is retarded at the temperatures below 18°C. Lower temperatures completely inhibit exogenous vitellogenesis. No yolky oocytes are found and only small oocytes are discernible in the ovary (Fishelson, 1966). The reproductive activity is only initiated when the water temperature increases above 22°C (Fishelson, 1966; Jalabert and Zohar, 1982).

In equatorial and tropical regions, the variations of temperature and photoperiod are less than in the subtropical regions. Therefore, in the equatorial region *O. niloticus* in spawning condition can be found throughout the year. Numbers of spawning fish, however, may vary slightly



with rainfall; higher spawning activity may coincide with the wet season (Lowe-McConnell, 1959; De Vlaming, 1974 and Jalabert and Zohar, 1982).

#### 5.1.4. Maturity and Spawning in Response to Internal Stimuli

As in other vertebrates, there are numerous glands and hormones involved, either directly or indirectly with the regulation of the fish reproductive system (Fontaine, 1976). The receptors which are stimulated by external factors such as a rise in temperature convey these environmental stimuli to the brain as a series of neural inputs. This information reaches the hypothalamus and causes the release of hypothalamic peptides, known as releasing hormones. The releasing hormones may accompany sexual maturity by stimulating the pituitary gland to release gonadotropic hormones (GtH), which in turn act on the gonads. The gonads subsequently produce the appropriate sex steroid hormones, which accelerates gametogenesis and regulates the development of secondary sexual characters, i.e., nuptial coloration and breeding behaviour. The levels of GtH in the pituitary gland and in the blood, increase during the breeding season. This general pattern has been demonstrated for goldfish, rainbow trout and tench (Breton, Billard, Jalabert and Kann, 1972; Breton, Jalabert, Fostier and Billard, 1975), salmonids (Crim, Meyer and Donaldson, 1973, Crim, Watts and Evans, 1975), brown trout Billard et



al., (1978), in plaice, *Pleuronectes platessa* (Wingfield and Grimm, 1977), winter flounder, *Pseudopleuronectes americanus* (Campbell et al., 1976), cichlid, *O. aureus* (Yaron, Terkatin-Shimony, Shaham and Salzer, 1977).

Prolactin is another pituitary hormone which controls the physiological processes, such as, osmoregulation, growth, metabolism and reproduction of teleosts (Hirano, 1986). Under natural spawning conditions, ovarian development in tilapias may be inhibited during parental care. On the other hand, Tan, Wong, Pang and Lam (1988) suggested that prolactin causes the augmentation of the gonadotropin-controlled synthesis of oestradiol-17 $\beta$  through granulosa cells of guppy, *Poecilia reticulata*, ovary.

Steroid hormones are mainly produced in the gonads. Oestradiol-17 $\beta$  ( $E_2$ ) is a common steroid found in teleost ovaries. In *O. aureus* ovary, the  $E_2$  has been found to control the synthesis of vitellogenin in the liver and the mobilization of mineral and fat stores (Katz and Eckstein, 1974). A high level of  $E_2$  produced by the gonads has also been reported to increase during the ovarian development of trout (Lambert et al., 1978), carp, *Cyprinus carpio* (Santos et al., 1986) and goldfish (Kobayashi et al., 1986). The  $E_2$  levels are low during endogenous vitellogenesis and increase at the beginning of exogenous vitellogenesis. This increase continues throughout the period of exogenous yolk formation and reaches a maximum some weeks before spawning.



In addition, Katz and Eckstein (1974) also reported that the levels of testosterone (T), 11-ketotestosterone and 11-deoxycorticosterone were considerably higher in the plasma of female *O. aurea* kept at 30°C than those fish kept at 18°C. Moreover, Yaron et al. (1977) reported a positive correlation of E<sub>2</sub> and ovarian weight in *O. aureus*. They also reported that during final oocyte maturation, the levels of GtH and E<sub>2</sub> increased markedly and ovulation occurred when the E<sub>2</sub> levels had decreased. This reports suggested a negative feedback of E<sub>2</sub> similar to that found in higher vertebrates (Whitehead et al., 1978a,b).

The age and size at which tilapia mature, can be variable and mature fish as young as 2 to 3 months have been reported (Jalabert and Zohar, 1982; Pullin, 1983; Eyeson, 1983). The age structure of these tilapia groups, however, is often unknown and therefore small mature fish may, in fact, be old.

## 5.2 OBJECTIVES

This study investigated the early gonadal development, maturity and accompanying steroid profiles during sexual differentiation and puberty in hatchery reared *O. niloticus*.



### 5.3 METHODS

The experimental fish in this investigation were obtained as described in section 2.2.1.

#### 5.3.1 Effect of Stocking Density on the Timing of Sexual Differentiation of *O. niloticus* (Experiment 1)

A batch of *O. niloticus* eggs from an ovulated female was manually stripped and fertilized with milt pooled from two males. The fertilized eggs were incubated in an upwelling round-bottomed hatching jar (Rana, 1986) until 5 days post-hatch. The fry were then transferred into 20 l plastic aquaria and maintained at  $27 \pm 1^\circ\text{C}$  in a closed recirculatory water system. The fry were stocked at 1) low (2 fry/litre), 2) medium (10 fry/litre) and 3) high densities (20 fry/litre) and fed in excess 5 times a day with ground trout pellets containing 54% protein.

The fry were randomly sampled at day 1 post-hatched fry. Sixteen, 20 and 40 fry from each density, respectively, were randomly sampled every 2 - 3 days until 42 days old and sacrificed in an overdose of benzocaine. Total length and body weight of each fry were recorded and the middle section of the bodies were dissected and then fixed in Bouin's fluid and processed in paraffin wax (section 2.2.1) for histological examination. Serial cross sections were cut at 5  $\mu\text{m}$  thickness, stained with Heidenhain's



haematoxylin (section 2.2.1.2) and examined under the microscope (Figure 2.1). Sexual differentiation of the fry was detected by the presence of gonadal cells in meiotic prophase (leptotene).

### 5.3.2 Gonadal Development and Steroid Profiles of *O. niloticus* During Puberty (Experiment 2)

Three ovulated *O. niloticus* females were manually stripped and each batch of eggs was fertilized with the milt from 2 males. The fertilized eggs were then incubated separately in the upwelling round-bottomed jars (Rana, 1986). The 5 days old fry were transferred into 3 (1x1x0.3m) fibre glass tanks and stocked at the density of 2 fry per litre to avoid early sex differentiation (experiment 1).

During the first month, the fry were fed with ground commercial trout pellet containing 54% protein 3 - 4 times a day. Thereafter they were fed with trout pellet no. 3 (54% protein) 2 - 3 times a day for 3 months and pellet no. 4 (40 - 42% protein) twice a day until the end of the study. At 12 weeks the fish were hand sexed and held separately in hapas suspended in the same tanks.

Fifteen to twenty fish from each tank were randomly sampled twice a month until all the females attained sexual maturity. The fish were killed in an overdose of benzocaine and their total length, body weight, liver weight and gonad



weight were recorded. Blood samples were collected from each fish and the serum from each sample was separated and stored at -20°C as described in section 2.1.3. The individual serum samples were analyzed for total calcium, testosterone and oestradiol-17 $\beta$  as described in section 2.4 and 2.5. The data of fish from each age group were pooled and presented as their mean $\pm$ SE.

Stereological studies were restricted to only female gonads. The ovaries were fixed in Bouin's fixative. The gonads from young females were processed with wax, whereas the mature female gonads were processed with historesin (section 2.2.1). Sections were stained with either haematoxylin and eosin or polychrome stain as described in section 2.2.1.2a,c and examined to identify the developmental stages of oocytes according to chapter 3 (Table 3.1) and the ovarian volume fractions of the different oocyte stages in each age group were determined according to section 2.3.



## 5.4 RESULTS

### 5.4.1 Effect of Stocking Density on Timing of Sexual Differentiation of *O. niloticus* (Experiment 1)

The length and weight of the newly hatched fry showed no significant differences ( $P>0.05$ ) between the three densities. The differences of the length and weight of fry at 18 days between the three stocking densities were significant ( $P<0.05$ ) (Figure 5.1).

Primordial germ cells (PGCs) and gonadal-sacs were observed in days 1 and 3 post-hatched fry, respectively. By 11 days post-hatching, 30% of the medium and 45% (Table 5.1) of the high density reared fry showed evidence of cells in meiotic prophase. In contrast, within the same time in the fry kept at low density, no sexual differentiated gonads were found.

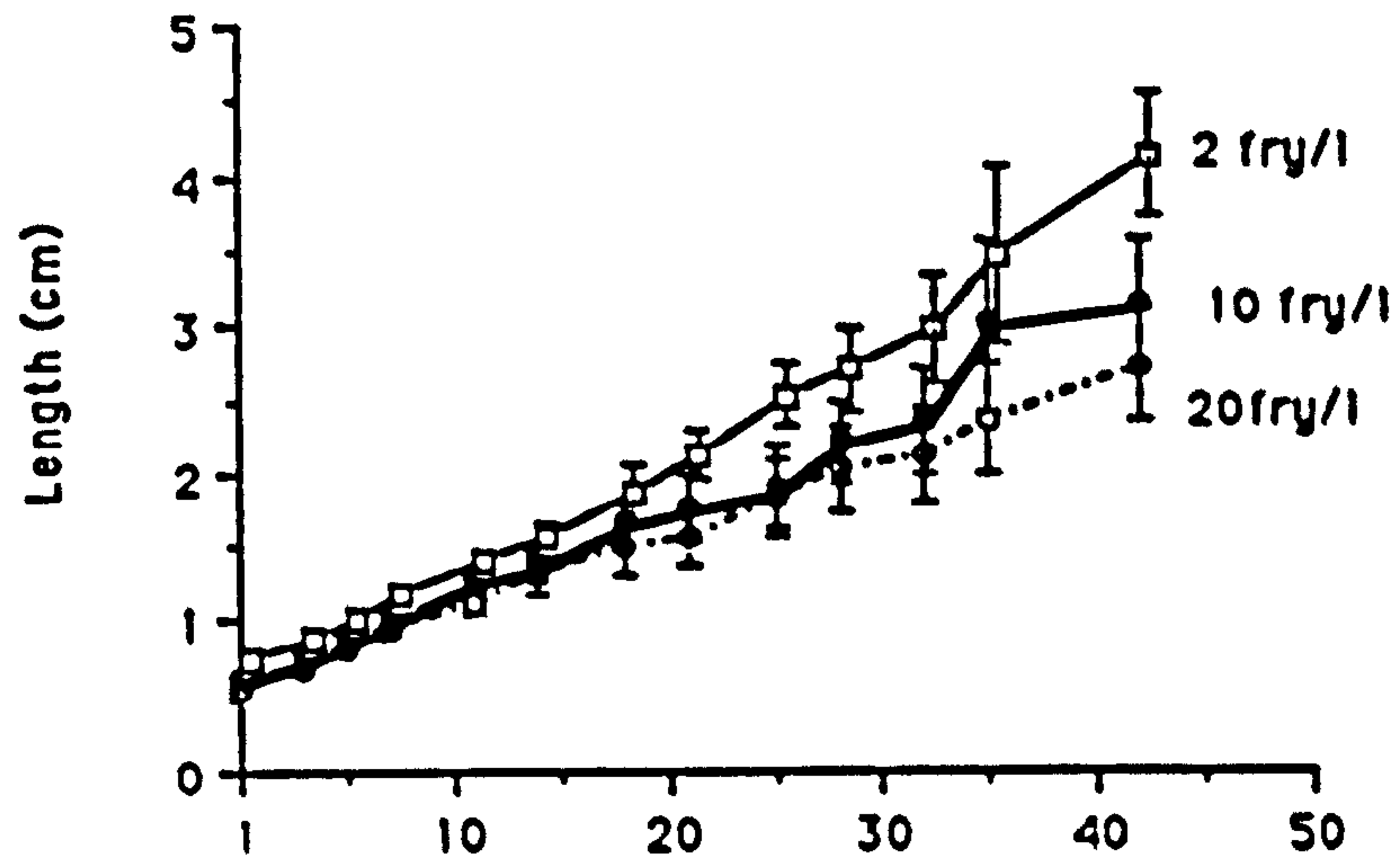
Table 5.1: Effect of stocking density on the mean ( $\pm$ SE) total length and weight of *O. niloticus* fry during the onset of sexual differentiation

| Stocking density<br>(fry/l) | Day 11         |                             |                             | Day 14         |                             |                             |
|-----------------------------|----------------|-----------------------------|-----------------------------|----------------|-----------------------------|-----------------------------|
|                             | sex dif<br>(%) | length<br>(mm)              | weight<br>(mg)              | sex dif<br>(%) | length<br>(mm)              | weight<br>(mg)              |
| 1. Low<br>(2)               | 0              | 12.2 $\pm$ 0.1 <sup>a</sup> | 26.2 $\pm$ 0.4 <sup>a</sup> | 100            | 13.7 $\pm$ 0.1 <sup>a</sup> | 37.9 $\pm$ 0.9 <sup>a</sup> |
| 2. Medium<br>(10)           | 30             | 12.0 $\pm$ 0.1 <sup>a</sup> | 22.7 $\pm$ 0.4 <sup>a</sup> | 100            | 13.1 $\pm$ 0.1 <sup>a</sup> | 32.0 $\pm$ 0.7 <sup>a</sup> |
| 3. High<br>(20)             | 45             | 11.2 $\pm$ 0.1 <sup>a</sup> | 20.3 $\pm$ 0.5 <sup>a</sup> | 100            | 13.0 $\pm$ 0.1 <sup>a</sup> | 30.0 $\pm$ 1.3 <sup>a</sup> |

Means with the same letters within columns indicate no significant ( $P>0.05$ ) differences.



(a)



(b)

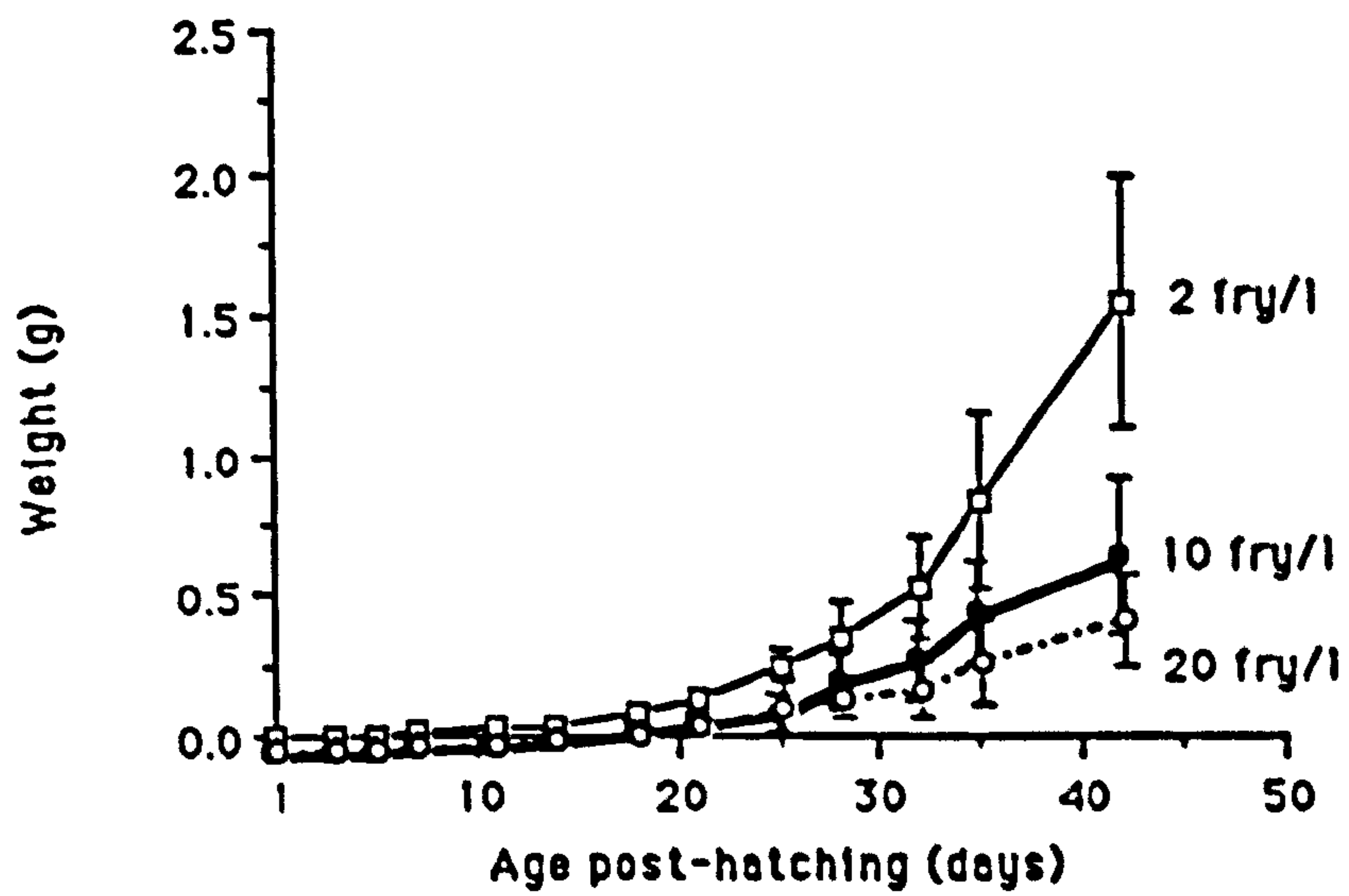


Figure 5.1: Effect of stocking densities on growth : (a) total length, (b) body weight of *O. niloticus* fry initial stocked at three stocking densities: 2, 10 and 20 fry/l.



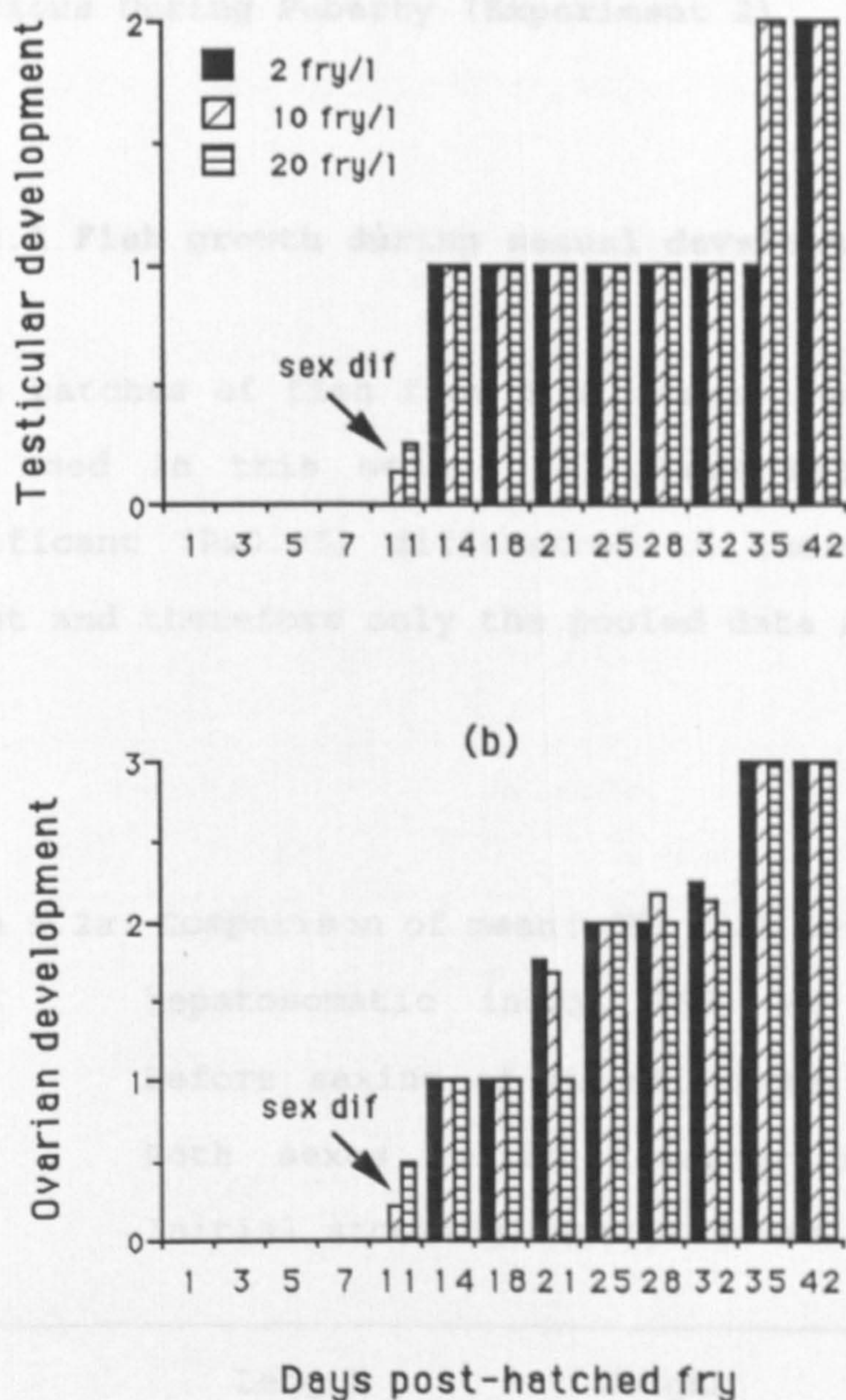


Figure 5.2: Onset of sexual differentiation during gonadal development of *O. niloticus* fry.

(a) Testicular stages (Y axis): 1) Primary spermatocyte in meiotic prophase (leptotene); 2) Secondary spermatocyte.

(b) Ovarian stages (Y axis): 1) Primary oocyte in meiotic prophase (leptotene), 2) Chromatin nucleolus oocyte (st1), 3) Perinucleolus oocyte (st2).

Arrow indicates occurrence of sexually differentiated gonads.



## 5.4.2 Gonadal Development and Steroid Profiles of *O. niloticus* During Puberty (Experiment 2)

### 5.4.2.1 Fish growth during sexual development

Three batches of fish from 3 different female-broodstocks were used in this study. All these batches showed no significant ( $P>0.05$ ) differences in their total length, weight and therefore only the pooled data are given (Table 5.2a).

Table 5.2a: Comparison of mean( $\pm$ SE) fish length, weight and hepatosomatic index (HSI) at different ages before sexing of *O. niloticus* during puberty. Both sexes reared together in 1 m<sup>2</sup> tanks. Initial stocking densities was 2 fry/l.

| Age<br>(wks) | Length<br>(cm) | Weight<br>(g)  | HSI<br>(%)     |
|--------------|----------------|----------------|----------------|
| 2            | 1.0 $\pm$ 0.11 | 0.2 $\pm$ 0.01 | -              |
| 4            | 2.1 $\pm$ 0.11 | 0.2 $\pm$ 0.04 | 5.3 $\pm$ 0.33 |
| 6            | 3.4 $\pm$ 0.14 | 0.9 $\pm$ 0.14 | 3.6 $\pm$ 0.26 |
| 8            | 4.6 $\pm$ 0.26 | 1.7 $\pm$ 0.27 | 3.3 $\pm$ 0.21 |
| 10           | 6.4 $\pm$ 0.34 | 4.5 $\pm$ 0.90 | 3.2 $\pm$ 0.27 |



The weight of the males and females of the same age were significantly ( $P < 0.05$ ) different (Table 5.2 b) at 18 weeks. By 22 weeks these differences were found in female length.

Table 5.2b: Comparison of the male and female mean ( $\pm$ SE) total length, weight and HSI at different ages of *O.niloticus* during puberty. The sexes were separated and the fish were kept in hapas suspended in 1 m<sup>2</sup> tanks.

| Age<br>(wks) | Length (cm)                             |   | Weight (g)                              |   | HSI (%)                                |  |
|--------------|---|---|---|---|--|--|
|              | male                                    | female                                  | male                                    | female                                  | male                                   | female                                 |
| 12           | 7.3 $\pm$ 0.43 <sup>a1</sup><br>(n=8)   | 5.1 $\pm$ 0.65 <sup>a1</sup><br>(n=20)  | 9.3 $\pm$ 1.81 <sup>a1</sup><br>(n=8)   | 11.0 $\pm$ 2.71 <sup>a1</sup><br>(n=20) | 3.4 $\pm$ 0.43 <sup>a1</sup><br>(n=8)  | 3.6 $\pm$ 0.33 <sup>a1</sup><br>(n=20) |
| 14           | 9.2 $\pm$ 0.62 <sup>a1</sup><br>(n=22)  | 8.9 $\pm$ 0.64 <sup>b1</sup><br>(n=21)  | 16.1 $\pm$ 2.73 <sup>a1</sup><br>(n=22) | 16.1 $\pm$ 3.72 <sup>a1</sup><br>(n=21) | 2.7 $\pm$ 0.11 <sup>a1</sup><br>(n=22) | 2.7 $\pm$ 0.13 <sup>a1</sup><br>(n=21) |
| 16           | 12.2 $\pm$ 1.72 <sup>b1</sup><br>(n=20) | 9.7 $\pm$ 0.57 <sup>b1</sup><br>(n=25)  | 26.7 $\pm$ 4.63 <sup>b1</sup><br>(n=20) | 19.7 $\pm$ 3.32 <sup>a1</sup><br>(n=25) | 2.4 $\pm$ 0.24 <sup>a1</sup><br>(n=20) | 2.9 $\pm$ 0.32 <sup>a1</sup><br>(n=25) |
| 18           | 12.0 $\pm$ 0.02 <sup>b1</sup><br>(n=21) | 11.0 $\pm$ 0.45 <sup>b1</sup><br>(n=23) | 36.3 $\pm$ 5.12 <sup>b2</sup><br>(n=21) | 26.4 $\pm$ 3.50 <sup>b1</sup><br>(n=23) | 2.2 $\pm$ 0.15 <sup>a2</sup><br>(n=21) | 2.5 $\pm$ 0.18 <sup>a1</sup><br>(n=23) |
| 20           | 12.5 $\pm$ 0.50 <sup>b1</sup><br>(n=29) | 12.2 $\pm$ 0.32 <sup>b1</sup><br>(n=31) | 42.3 $\pm$ 4.98 <sup>b2</sup><br>(n=29) | 39.1 $\pm$ 3.49 <sup>b1</sup><br>(n=31) | 2.6 $\pm$ 0.11 <sup>a1</sup><br>(n=29) | 3.1 $\pm$ 0.14 <sup>b2</sup><br>(n=31) |
| 22           | 14.7 $\pm$ 0.45 <sup>b2</sup><br>(n=30) | 14.1 $\pm$ 0.39 <sup>b1</sup><br>(n=30) | 70.2 $\pm$ 6.07 <sup>b2</sup><br>(n=30) | 57.3 $\pm$ 4.83 <sup>b1</sup><br>(n=30) | 2.6 $\pm$ 0.12 <sup>a1</sup><br>(n=30) | 3.3 $\pm$ 0.14 <sup>b2</sup><br>(n=30) |
| 24           | 16.4 $\pm$ 0.41 <sup>b2</sup><br>(n=30) | 15.5 $\pm$ 0.40 <sup>b1</sup><br>(n=30) | 94.0 $\pm$ 7.43 <sup>b2</sup><br>(n=30) | 77.5 $\pm$ 4.96 <sup>b1</sup><br>(n=30) | 2.3 $\pm$ 0.14 <sup>a1</sup><br>(n=30) | 2.7 $\pm$ 0.09 <sup>b2</sup><br>(n=30) |

Means with the same letters and numbers within columns and rows of parameters, respectively, indicate no significant ( $P > 0.05$ ) differences.



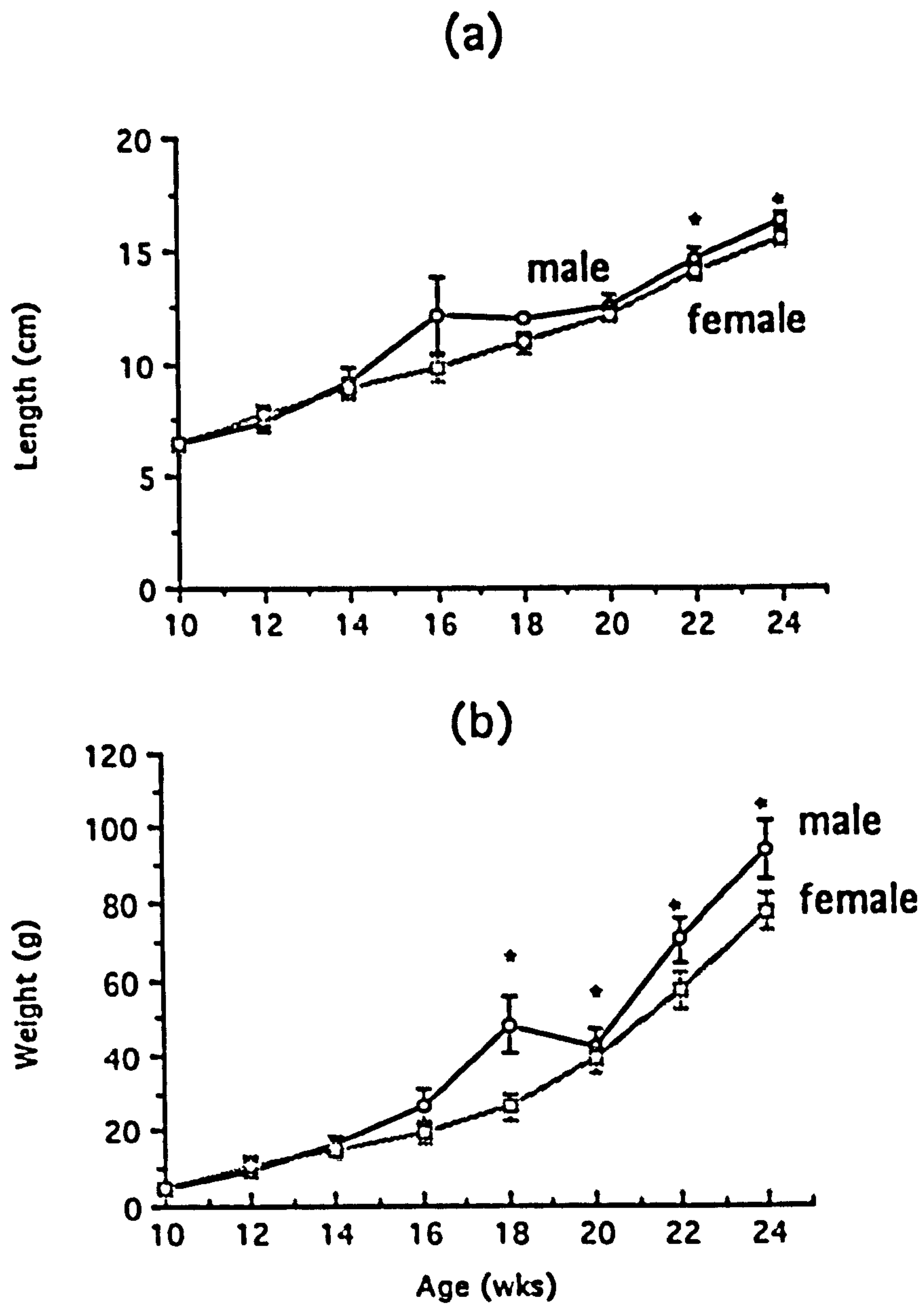


Figure 5.3: Average growth: (a) total length and (b) weight of *O. niloticus* during puberty. Numbers of fish examined at each sampling age were 15-30 fish. '\*' indicates significant ( $P < 0.05$ ) differences between males and females of the same age. Means based on data pooled from the three batches of fish used in the present study.



#### 5.4.2.2 Sexual development during puberty

##### a. Hepatosomatic index (HSI)

The HSI of the males ranged between 2.2 to 3.4% (Table 5.2b) and there were no significant ( $P>0.05$ ) differences between the HSI at every age throughout the study. The HSI of the females in comparison, increased significantly ( $P<0.05$ ) at 20 (3.1%) and 22 weeks (3.3%) but then decreased significantly ( $P<0.05$ ) at 24 weeks (2.7%) (Table 5.2b).

##### b. Gonadal development and GSI

###### Males

The GSI of the males ranged from 0.2 to 0.7%, and were not significantly ( $P>0.05$ ; Table 5.3a) different throughout the study. The results of gonadal maturation from stereological analysis showed that at 12 weeks, 10.9% of the male testes (GSI = 0.2%) contained a few metamorphosed spermatozoa. The percentage of mature males with testes containing spermatozoa rapidly increased to 92% and 100% at 14 (GSI = 0.2%) and 16 weeks (GSI = 0.3%), respectively (Figure 5.4). In addition, at 16 weeks, males showed secondary sexual characteristics such as purple-red heads.



## Females

The increase in female GSIs at 20 weeks (2%) were significantly ( $P < 0.05$ ; Table 5.3b) greater than those at 18 weeks (0.5%).

Stereological analysis was used to chart the chronological ovarian volume fractions of different oocyte stages (Table 5.4; Figure 5.5). The oogonia developed into stage 2 oocytes (previtellogenic oocytes) at 6 weeks and were arrested at this stage until 14 weeks (GSI = 0.4%). At 16 weeks the oocytes (GSI = 0.3%) continued their development into stage 3 (endogenous vitellogenesis), stages 4, 5 (exogenous vitellogenesis) and subsequently developed into stage 6 oocytes (mature oocyte) at 18 weeks (GSI = 0.5%). By 20 weeks the female GSI reached 2% and 71% of their ovaries containing 46.7% stage 6 oocytes (Figure 5.4). The female GSI continued the increase to 2.9 and 3.6% by 22 and 24 weeks, respectively (Figure 5.5). At 22 and 24 weeks, their ovaries contained 71.8 and 67.5% stage 6 oocytes as well as 4.6 and 6% of atretic oocytes, respectively (Table 5.4). These values at 22 and 24 weeks, however, were not significantly ( $P > 0.05$ ) different.



### **c. Hormonal levels during puberty**

#### **Males**

Levels of  $\text{Ca}^{2+}$  and  $\text{E}_2$  in males during 14 to 18 weeks, were slightly less than the females of the same age. The T levels of males were higher than the female at the same ages (Table 5.3a). All the hormone levels increased with no significant ( $P>0.05$ ) differences being found between 18 and 20 weeks. The levels of  $\text{Ca}^{2+}$  (12.8mg%) and T (65.5 ng/ml) increased significantly ( $P<0.05$ ) by 22 weeks and these levels were maintained at 12.8 mg% for  $\text{Ca}^{2+}$  and 73.2 ng/ml for T at 24 weeks. The  $\text{E}_2$  levels of males increased to 11.3 and 21.5 ng/ml at 20 weeks and 22 weeks, respectively (Figure 5.6), and the levels showed no significant ( $P>0.05$ ) differences during these periods. Subsequently, the levels dropped significantly ( $P<0.05$ ) by 24 weeks to 2.7ng/ml. By 22 weeks the males had attained sexual maturity.

#### **Females**

In 14 - 18 week old females, the levels of  $\text{Ca}^{2+}$ ,  $\text{E}_2$  and T were similar ( $P>0.05$ ) between the different female age groups. These levels were 12.6 - 15.7mg%, 4.5 - 6.6ng/ml and 28.8 - 31.5ng/ml for  $\text{Ca}^{2+}$ ,  $\text{E}_2$  and T, respectively (Table 5.3b). The significantly ( $P<0.05$ ) higher levels of  $\text{Ca}^{2+}$ ,  $\text{E}_2$  and T were first detected at 20 weeks (Table 5.3b). These levels continued to increase by 22 weeks ( $\text{Ca}^{2+}$  = 34.6 mg%;



$E_2$  = 26.7 ng/ml and T = 43.4ng/ml) and only the levels of  $Ca^{2+}$  and  $E_2$  subsequently dropped at 24 weeks ( $Ca^{2+}$  = 28.6 mg% and  $E_2$ =19.6 ng/ml). In contrast, the level of T continued to increase until 24 weeks (52.2 ng/ml) (Table 5.3b and Figure 5.6). These peaks corresponded with the maximum stage 6 oocyte volume fraction (71.8%; Table 5.4).

Table 5.3a: Mean ( $\pm$ SE) gonadosomatic index (GSI), total calcium ( $Ca^{2+}$ ), testosterone (T) and 17 $\beta$ -oestradiol ( $E_2$ ) of maturing *O. niloticus* male reared in captivity.

| Age (wks) | GSI (%)                               | Total calcium (mg%)                    | Testosterone (ng/ml)                   | 17 $\beta$ -oestradiol (ng/ml)         |
|-----------|---------------------------------------|--|--|--|
| 14        | 0.2 $\pm$ 0.07 <sup>a</sup><br>(n=6)  | 10.4 $\pm$ 0.57 <sup>a</sup><br>(n=15) | 37.8 $\pm$ 7.06 <sup>a</sup><br>(n=11) | 5.4 $\pm$ 1.35 <sup>a</sup><br>(n=11)  |
| 16        | 0.3 $\pm$ 0.10 <sup>a</sup><br>(n=17) | 10.6 $\pm$ 0.74 <sup>a</sup><br>(n=20) | 50.1 $\pm$ 9.23 <sup>a</sup><br>(n=13) | 4.3 $\pm$ 0.73 <sup>a</sup><br>(n=13)  |
| 18        | 0.3 $\pm$ 0.10 <sup>a</sup><br>(n=21) | 10.8 $\pm$ 0.66 <sup>a</sup><br>(n=21) | 36.2 $\pm$ 7.67 <sup>a</sup><br>(n=11) | 2.7 $\pm$ 0.72 <sup>a</sup><br>(n=11)  |
| 20        | 0.4 $\pm$ 0.12 <sup>a</sup><br>(n=21) | 11.7 $\pm$ 0.41 <sup>a</sup><br>(n=28) | 43.7 $\pm$ 15.5 <sup>a</sup><br>(n=14) | 11.3 $\pm$ 4.13 <sup>a</sup><br>(n=12) |
| 22        | 0.4 $\pm$ 0.09 <sup>a</sup><br>(n=30) | 12.8 $\pm$ 0.38 <sup>b</sup><br>(n=30) | 65.5 $\pm$ 23.4 <sup>b</sup><br>(n=14) | 21.5 $\pm$ 9.95 <sup>a</sup><br>(n=13) |
| 24        | 0.7 $\pm$ 0.13 <sup>a</sup><br>(n=30) | 12.8 $\pm$ 0.37 <sup>b</sup><br>(n=30) | 73.2 $\pm$ 7.91 <sup>b</sup><br>(n=29) | 2.7 $\pm$ 0.62 <sup>b</sup><br>(n=8)   |

Means with the same letters within columns indicate no significantly ( $P>0.05$ ) differences.



Table 5.3b: Mean ( $\pm$ SE) of gonadosomatic index (GSI), total calcium ( $\text{Ca}^{2+}$ ), testosterone (T) and  $17\beta$ -oestradiol ( $\text{E}_2$ ) of maturing female *O.niloticus* reared in captivity.

| Age<br>(wks) | GSI<br>(%)                 | Total calcium<br>(mg%)      | Testosterone<br>(ng/ml)     | $17\beta$ -oestradiol<br>(ng/ml) |
|--------------|----------------------------|-----------------------------|-----------------------------|----------------------------------|
| 14           | $0.4 \pm 0.21^a$<br>(n=19) | $12.6 \pm 3.36^a$<br>(n=12) | $28.8 \pm 7.56^a$<br>(n=5)  | $4.5 \pm 0.95^a$<br>(n=5)        |
| 16           | $0.3 \pm 0.12^a$<br>(n=24) | $14.4 \pm 2.65^a$<br>(n=22) | $36.2 \pm 8.08^a$<br>(n=14) | $8.4 \pm 2.35^a$<br>(n=14)       |
| 18           | $0.5 \pm 0.23^a$<br>(n=23) | $15.7 \pm 3.10^a$<br>(n=22) | $31.5 \pm 6.79^a$<br>(n=15) | $6.6 \pm 1.88^a$<br>(n=15)       |
| 20           | $2.0 \pm 0.48^b$<br>(n=31) | $25.6 \pm 3.51^b$<br>(n=23) | $43.9 \pm 9.14^b$<br>(n=23) | $19.3 \pm 5.44^b$<br>(n=23)      |
| 22           | $2.9 \pm 0.56^b$<br>(n=30) | $34.6 \pm 5.69^b$<br>(n=30) | $43.4 \pm 5.74^b$<br>(n=27) | $26.7 \pm 8.41^b$<br>(n=27)      |
| 24           | $3.6 \pm 0.38^b$<br>(n=30) | $28.6 \pm 3.43^b$<br>(n=30) | $52.2 \pm 4.85^b$<br>(n=30) | $19.6 \pm 3.95^b$<br>(n=17)      |

Means with the same superscripts within columns indicate no significant ( $P > 0.05$ ) differences.



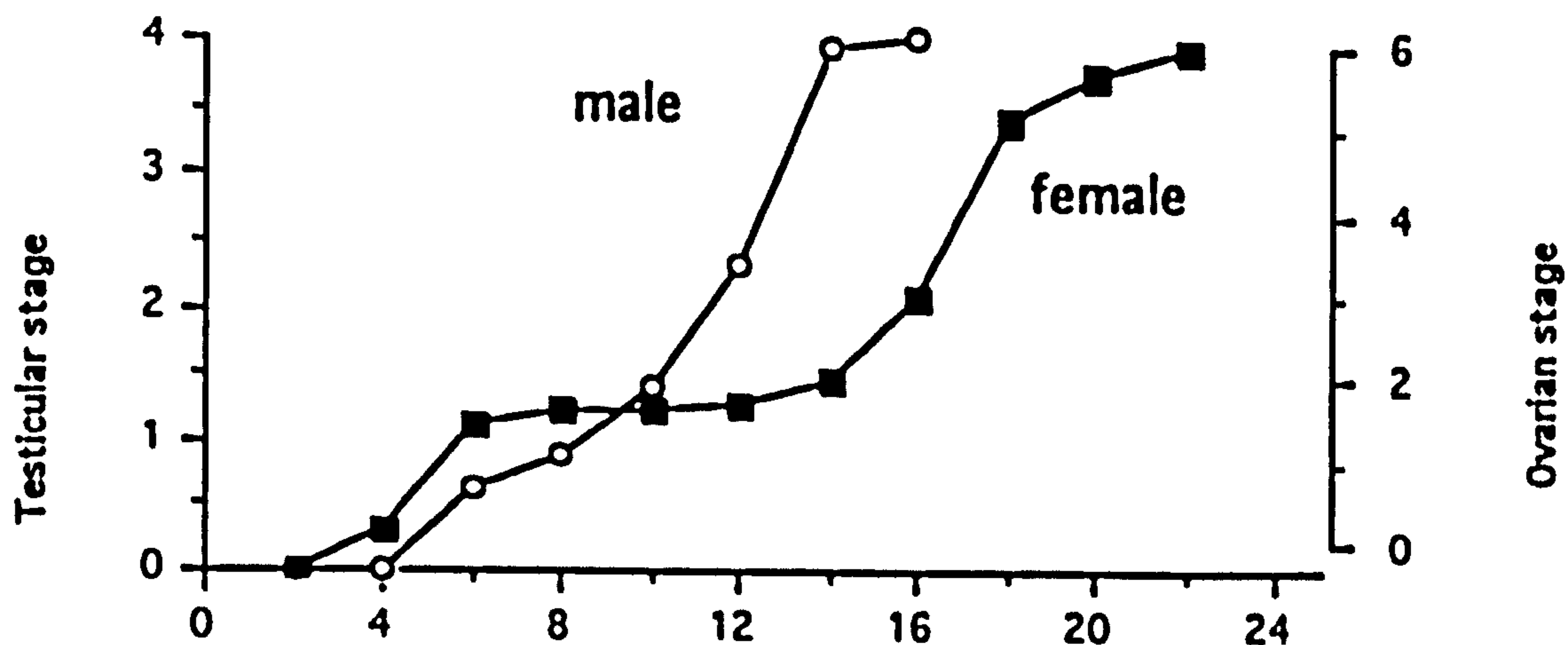
Table 5.4: Changes in the ovarian volume fractions (mean $\pm$ SE) of different ages of puberty *O. niloticus* in captivity.

| Age<br>(wks) | Ovarian volume fractions (%)           |  |                                       |  |  |                                       |
|--------------|--|--|---------------------------------------|--|--|---------------------------------------|
|              | stage2                                 | stage3                                 | stage4                                | stage5                                 | stage6                                 | Atresia                               |
| 12           | 72.8 $\pm$ 3.97 <sup>a</sup><br>(n=17) | 4.6 $\pm$ 2.02 <sup>a</sup><br>(n=17)  | 2.8 $\pm$ 1.92 <sup>a</sup><br>(n=17) | -                                      | -                                      | -                                     |
| 14           | 70.0 $\pm$ 4.79 <sup>a</sup><br>(n=18) | 4.8 $\pm$ 1.29 <sup>a</sup><br>(n=18)  | 2.5 $\pm$ 1.70 <sup>a</sup><br>(n=18) | 2.3 $\pm$ 1.94 <sup>a</sup><br>(n=18)  | 2.9 $\pm$ 2.92 <sup>a</sup><br>(n=18)  | -                                     |
| 16           | 77.7 $\pm$ 4.80 <sup>a</sup><br>(n=19) | 7.3 $\pm$ 3.68 <sup>a</sup><br>(n=19)  | 2.7 $\pm$ 1.18 <sup>a</sup><br>(n=19) | 1.2 $\pm$ 0.90 <sup>a</sup><br>(n=19)  | 1.9 $\pm$ 1.55 <sup>a</sup><br>(n=19)  | -                                     |
| 18           | 59.3 $\pm$ 5.99 <sup>a</sup><br>(n=18) | 10.6 $\pm$ 1.82 <sup>a</sup><br>(n=18) | 5.0 $\pm$ 1.44 <sup>a</sup><br>(n=18) | 3.4 $\pm$ 1.27 <sup>a</sup><br>(n=18)  | 5.3 $\pm$ 2.66 <sup>a</sup><br>(n=18)  | -                                     |
| 20           | 27.1 $\pm$ 5.49 <sup>b</sup><br>(n=20) | 9.9 $\pm$ 1.66 <sup>a</sup><br>(n=20)  | 3.9 $\pm$ 0.64 <sup>a</sup><br>(n=20) | 4.2 $\pm$ 0.82 <sup>a</sup><br>(n=20)  | 46.7 $\pm$ 6.71 <sup>b</sup><br>(n=20) | -                                     |
| 22           | 6.0 $\pm$ 0.64 <sup>b</sup><br>(n=20)  | 4.9 $\pm$ 0.57 <sup>a</sup><br>(n=20)  | 3.6 $\pm$ 0.49 <sup>a</sup><br>(n=20) | 10.2 $\pm$ 1.09 <sup>b</sup><br>(n=20) | 71.8 $\pm$ 2.27 <sup>b</sup><br>(n=20) | 4.6 $\pm$ 1.63 <sup>a</sup><br>(n=20) |
| 24           | 5.7 $\pm$ 1.06 <sup>b</sup><br>(n=17)  | 4.3 $\pm$ 0.49 <sup>a</sup><br>(n=17)  | 4.0 $\pm$ 0.49 <sup>a</sup><br>(n=17) | 11.8 $\pm$ 1.36 <sup>b</sup><br>(n=17) | 67.5 $\pm$ 2.93 <sup>b</sup><br>(n=17) | 6.0 $\pm$ 1.37 <sup>a</sup><br>(n=17) |

Means with the same letters within columns indicate no significant ( $P>0.05$ ) differences.



### (a) Gonadal development



### (b) Maturity of fish population

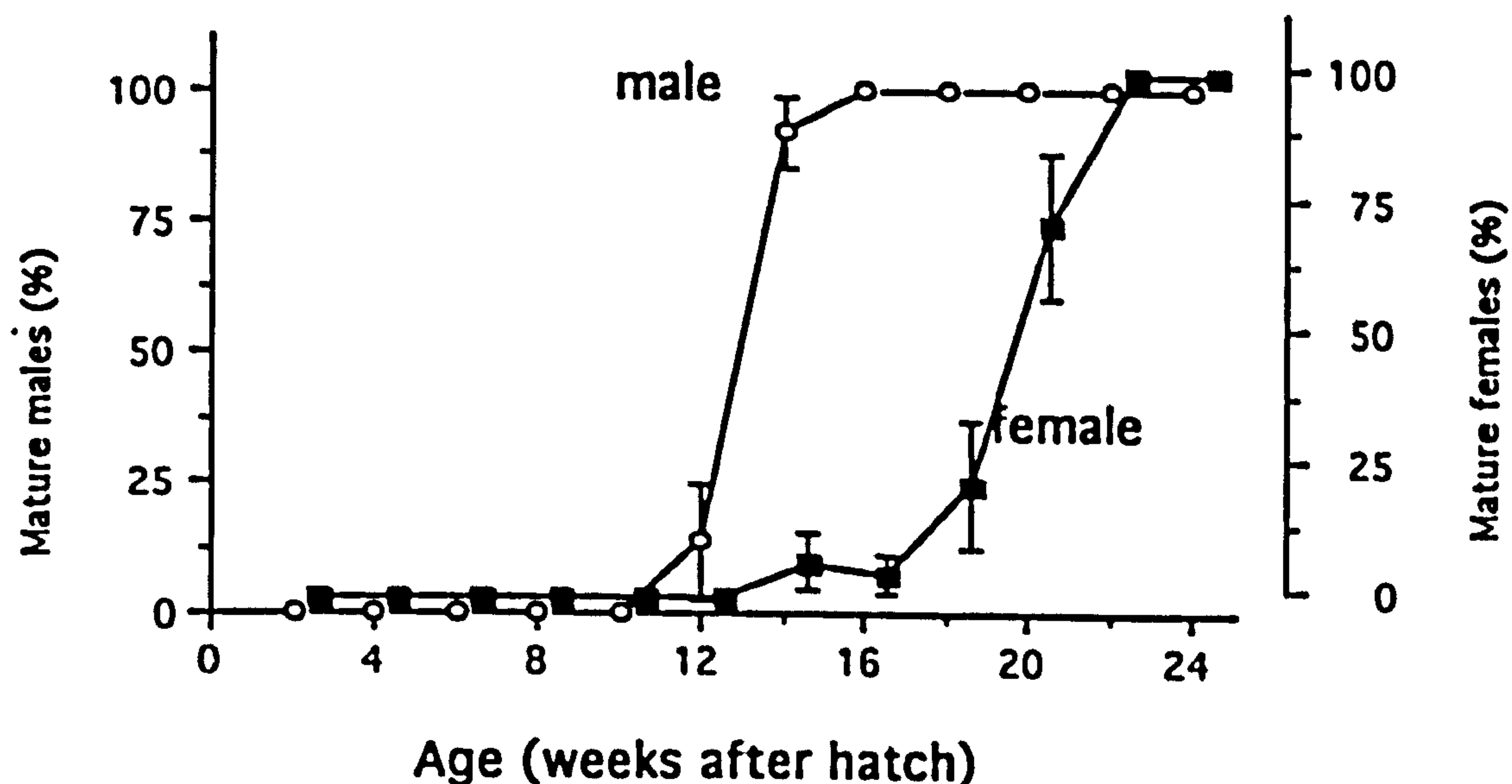


Figure 5.4 Relationship of gonadal development and maturity of male and female *O. niloticus* population at different ages during puberty.

(a) Gonadal development; Testicular stages (Y-axis):  
 1) Primary spermatocyte in meiotic prophase (leptotene);  
 2) Secondary spermatocyte; 3) Spermatid; 4) Spermatozoa;  
 Ovarian stages (Y-axis): 1) Chromatin nucleolus [stage 1];  
 2) Perinucleolus [stage 2], 3) Cortical alveoli [stage 3];  
 4) Stage 4; 5) Stage 5; 6) Stage 6.

(b) Maturity of fish population.



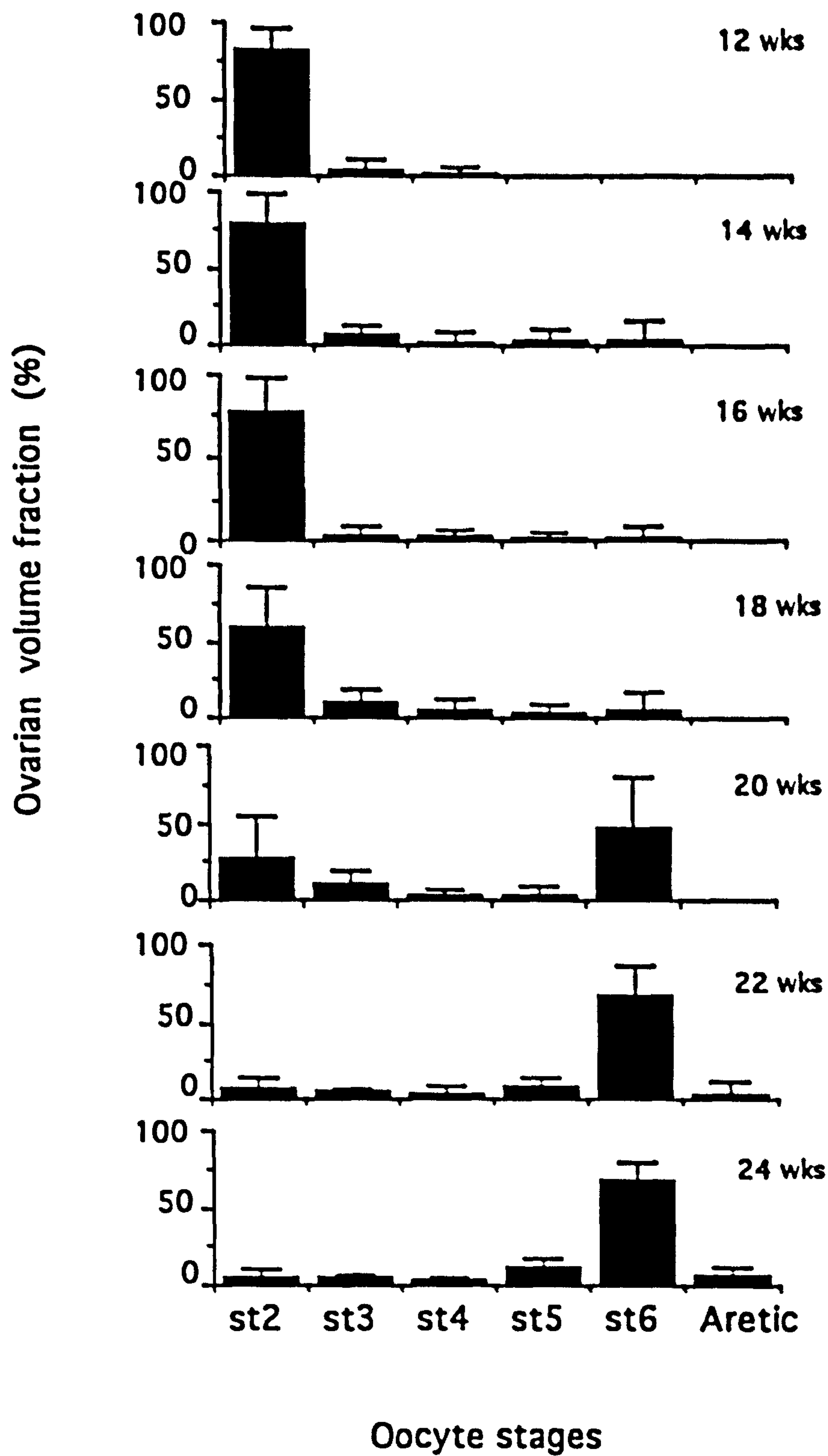


Figure 5.5 Progression of ovarian development in different ages of captive female *O. niloticus* during puberty.



Figure 5.6 Comparison of mean liver (HSI) and gonad (GSI) weights and the mean levels of serum hormones of *O. niloticus* males and females at different ages during puberty under captivity. Pooled data from individuals of the same age and sex.

- (a) Hepatosomatic index (HSI)
- (b) Gonadosomatic index (GSI)
- (c) Total calcium ( $\text{Ca}^{2+}$ )
- (d) Oestradiol-17 $\beta$  ( $\text{E}_2$ )
- (e) Testosterone (T).



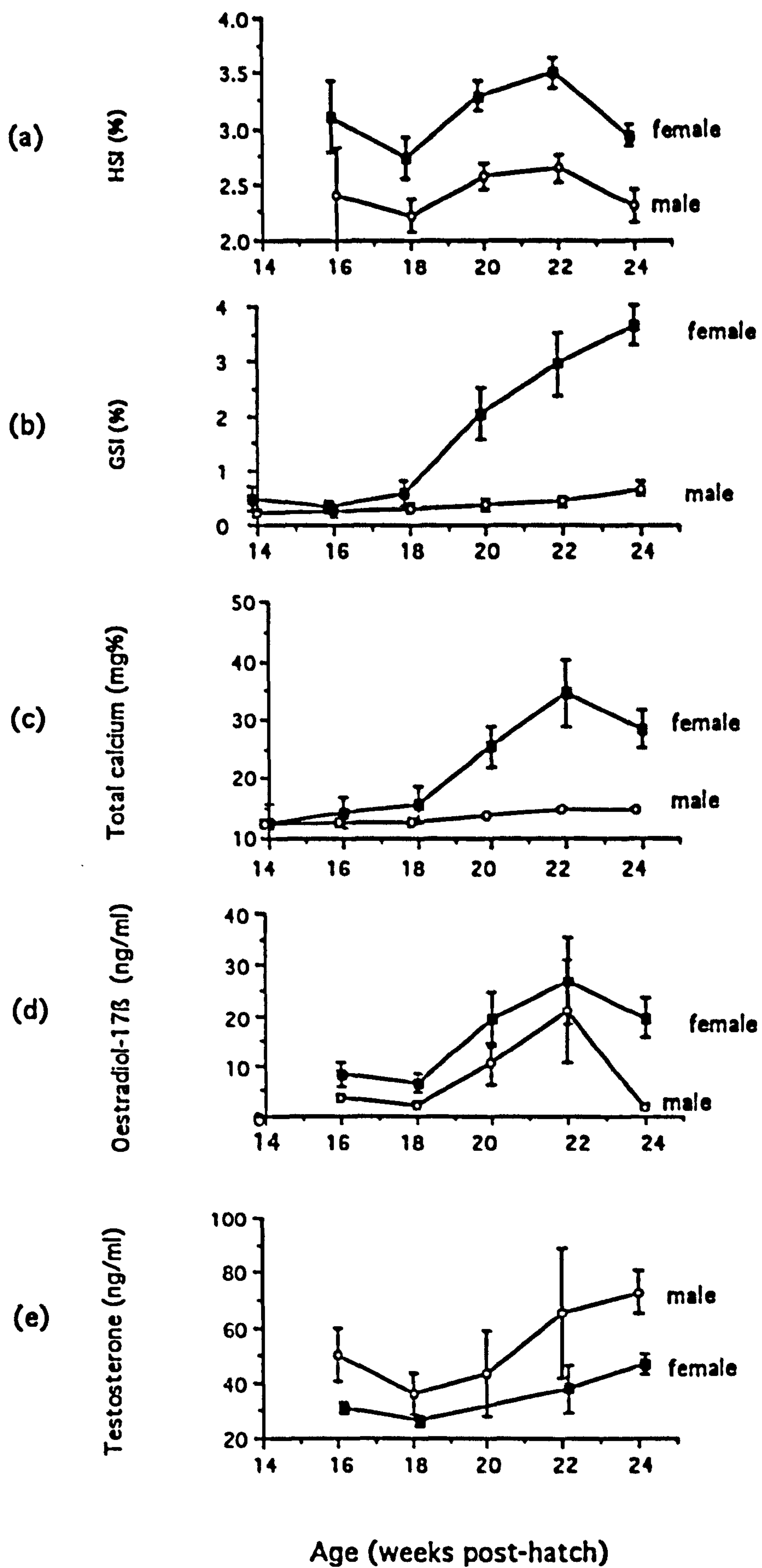


Figure 5.6



## 5.5 DISCUSSION

When the age of the fry is not known, the critical fry size of "less than 12 mm" has been advocated for steroid administration for practical sex reversal in various tilapia species (Tayamen and Shelton, 1978; Hopkins et al., 1979; Nakamura and Iwahashi, 1982). This criterion was similar to the size of the fry obtained in the low density (11 days post-hatched fry) rearing trials of this study. The fry were slightly longer than 12 mm (12.2mm) and at this fry size no gonads had differentiated (11 days post-hatching). In contrast, the length of the fry reared at the medium (12mm) and high (11.2mm) densities of the same age, were similar and shorter than 12 mm, but 30 and 45% of the fry, respectively, had completed the sexual differentiation. This present study showed that the rearing density affected the timing of onset of sexual differentiation. Therefore, if higher stocking densities (more than 10 fry/l) are used for hormonal sex reversal, the fry should be treated with steroid hormone either at/or earlier than 11 days post-hatching fry or at a size of less than 11 mm to ensure a high percentage of sex reversed *O. niloticus* males.



The testis of *O. niloticus* was of the lobule type and the arrangement of the seminiferous lobules were similar to those in percoid fishes (Hyder, 1970; Latif and Saady, 1973a). Under laboratory controlled conditions of the present study, spawning was not allowed. The male GSIs during 16 to 24 week period ranged from 0.3 - 0.7%. These GSIs are similar to that of the wild fish (0.1 - 0.8%) from the river Nile (Latif and Saady, 1973a) and the ripening (0.1 - 0.3%) and ripe (0.3 - 0.8%) testes of the same species from the White Nile (Babiker and Ibrahim, 1979a). The interstitial tissue is the source of testicular hormone (Hoar, 1955) or steroid production (Billard, Fostier, Weil and Breton, 1982). Nest building and courtship behaviours are controlled by androgen concentration from the preoptic brain area (Damski and Hornby, 1982). Both of the steroid sources release the steroid hormone into blood stream.

Throughout the present study levels of testosterone in the males were higher than the females. The T levels of the males gradually fluctuated during 16 to 20 weeks then showed a significant ( $P < 0.05$ ) increase at 22 weeks (65.5 ng/ml) and 24 weeks (73.2 ng/ml). Therefore, the males in the present study could attain courtship behaviour at 16 weeks at a size of 12.2 cm and 26.7 g. The progressive spawning behaviour which coincided with high T levels occurred at 22 weeks at a size larger than 14.7cm and 70.2g.



In females reared under similar conditions, no spawning was observed due to the high stocking density used in the tanks. The GSIs of the females significantly ( $P < 0.05$ ) increased from 0.5 (18 weeks) to 2% (20 weeks) and gradually increased to 2.9 (22 weeks) and 3.6% (24 weeks). Although the GSIs increased throughout the study, the ovarian volume fractions of stage 6 oocytes reduced at 24 weeks and the ovarian volume fraction of atretic oocytes gradually increased (Figure 5.5 and Table 5.4). The GSIs at 22 week old captive females (2.9%) corresponded to the ripe GSI (2.5%) in the wild of White Nile (Babiker and Ibrahim, 1979a).

During exogenous vitellogenesis, the liver synthesizes vitellogenin (high HSI) which is secreted into the bloodstream. (Hoar, 1955; Yaron et al., 1977; Whitehead et al., 1983). Levels of  $\text{Ca}^{2+}$  and  $\text{E}_2$  in female *O. niloticus* in this study gradually increased from 14 weeks (12.6 mg% of  $\text{Ca}^{2+}$ ; 4.5 ng/ml of  $\text{E}_2$ ) to 18 weeks (15.7 mg% of  $\text{Ca}^{2+}$ ; 6.6 ng/ml of  $\text{E}_2$ ) (Figure 5.6 and Table 5.3b). The  $\text{E}_2$  levels in the present study were similar to the levels of  $\text{E}_2$  (0.2 - 10 ng/ml) in intact pond cultured female *O. aureas* sampled during the spawning season (Yaron et al., 1977).

The histological and stereological results of the females in this study showed that by 20 weeks the ovary contained 46.7% of stage 6 oocytes and this increased to a peak of 71.8% at 22 weeks then reduced to 67.5% at 24 weeks (Table



5.4; Figure 5.5). These histological and stereological results corresponded to the  $Ca^{2+}$  and  $E_2$  levels which also peaked at 22 weeks (Figure 5.4, 5.5, 5.6) before decreasing at 24 weeks. The  $E_2$  profile in this study is similar to the  $E_2$  profile of brown trout, *Salmo trutta fario* and common carp, *Cyprinus carpio* (Billard et al., 1978), rainbow trout, *O. mykiss* (Scott et al., 1983; Whitehead et al., 1983) and goldfish (Kobayashi et al., 1986) which also showed a marked increase of GtH and  $E_2$  during the period of final oocyte maturation.

Under the captive conditions of this study, 100% of males (14.7 cm; 70.2g) and females (14.1 cm; 57.3g) matured by 22 weeks (5.5 months). The mature males were bigger than the mature females and these sizes at maturity were smaller than the wild sizes of the same species in the White Nile (male length = 15.7 cm; female length = 15 cm). The age of these wild fish, however, is unclear.



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## CHAPTER 6

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## 6. OVARIAN RECRUDESCENCE AND RELATED HORMONAL PROFILES DURING SPAWNING CYCLES OF POST-SPAWNED *Oreochromis niloticus* (L.) FEMALES.

### 6.1 INTRODUCTION

In nature, tilapias are seasonal breeders. Their breeding season which may last for about six months (McKaye, 1984; Hussein, 1984) often coincides with the optimum natural food availability to ensure high fry survival (McKaye, 1984). In comparison, under captive conditions, where the temperature is maintained at the optimal breeding temperature (27 - 30°C); tilapias can spawn throughout the year (Lowe-McConnell, 1959; Fishelson, 1966). The spawning frequency of individual females, however, may vary considerably and the factors which affect this variability between the individual females are unclear (Mires, 1982).

#### 6.1.1 Spawning Cycles of Teleosts

In teleost reproduction, the endogenous hormonal rhythms are synchronized with seasonal climatic changes. These changes stimulate gonadal development and finally ovulation via a series of interrelated hormonal triggers (de Vlaming, 1972; Bye, 1984).



Although the process of oocyte development is similar in most fish species, the total duration required for each spawning cycle is species dependant. In addition, in species such as the tilapias which have evolved complex courtship and spawning behaviour patterns; the spawning cycle may vary with fish size, species and type of parental care. For example, in *Oreochromis mossambicus*, females that rear their clutch in the buccal cavity (mouth-brooding females) showed spawning cycle of 40 days (Smith and Haley, 1987; 1988). When the clutch was removed (non-mouthbrooding females), the female spawning cycle was reduced to 25 days (Smith and Haley, 1987;1988). In addition, in *O. niloticus* non-mouthbrooding females, the spawning cycle was considerably shorter (Siraj et al., 1983). They reported that the spawning cycle of the first and second year fish of this same species, were 7 and 12 days, respectively. The reason for this variability of the spawning cycle particularly within species remains unclear and basic information on oocyte recrudescence of captive stock during spawning cycle is at best sparse.

#### 6.1.2 Morphological Structure and Hormonal Profiles

General morphological structures of oocytes during previtellogenic, vitellogenic and maturation (stage 6) stages are described in chapter 3. At maturity, stage 6 oocytes are characterised by the migration of germinal vesicle to the periphery of the oocytes, breakdown of



germinal vesicle and hydration and ovulation of the oocytes (Wallace and Selman, 1981; Nagahama, 1983; Wallace et al., 1987; Selman and Wallace, 1989).

Presence of some organelles such as smooth endoplasmic reticulum or tubular mitochondria in the follicle of oocytes indicates steroid synthesis, such as testosterone, oestradiol-17 $\beta$ . These steroids in ovary of fish can be determined by enzymatic activities (e.g., 3 $\beta$ -hydroxysteroid dehydrogenase; 3 $\beta$ -HSD) which increases during the spawning season (Fostier, Jalabert, Billard, Breton and Zohar, 1983). There are numerous sites in the ovary where these steroids are produced. Their locations have been identified with the help of histochemical techniques and electron microscopical studies (Kessel and Panje, 1968; Blum and Weber, 1968; Yaron, 1971; Nagahama, Chan and Hoar, 1976; Lam, Nagahama, Chan and Hoar, 1978; Kagawa and Takano, 1979; Lang, 1981; Nagahama and Kagawa, Young, Adachi and Nagahama, 1982; Nagahama, Kagawa, H. and Young, 1982). Using these techniques, it was concluded that granulosa cells, special thecal cells, corpora lutea, corpora atreca and interstitial gland cells are implicated in steroid synthesis.

The synthesis of steroids by the granulosa cells is accompanied by intracellular changes. Selman and Wallace (1983) and Selman, Wallace and Barr (1986) have shown that the granulosa cells of *Fundulus heteroclitus*, undergo



specific cytological alterations during the final oocyte maturation phase; these include a proliferation of the Golgi complexes and an increase in number of the cisternae of the granular endoplasmic reticulum and of free ribosomes. These changes suggest that the granulosa cells may produce the steroid that induces final oocyte maturation ( $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one;  $17\alpha,20\beta$ -P) (Scott and Canario, 1987).

Special thecal cells have also been involved in steroid synthesis. Ultrastructural studies of these cells have revealed advanced stages of mitochondrial tubular cristae and tubular agranula endoplasmic reticulum in the cytoplasm of these cell types (Nagahama, 1983).

In *O. mossambicus* for example, some smooth endoplasmic reticulum, Golgi complex vesicles, microvilli and microfilaments were found in the granulosa cells; while the active  $3\beta$ -hydroxysteroid dehydrogenase ( $3\beta$ -HSD) was found in thecal cells of 5-7 day post-spawned fish. The intense activity of  $3\beta$ -HSD was found at 10 days post-spawning. In females that were allowed to rear their clutch, atresia was initially observed in ovaries at 25 days post-spawning (Smith and Haley, 1987).



### 6.1.3 Hormonal Profiles of Teleosts During Spawning Cycle

Levels of hormones such as gonadotropins (GtHs), oestradiol-17 $\beta$  (E<sub>2</sub>), testosterone (T), progesterone, 17 $\alpha$ ,20 $\beta$ -P, etc; vary during spawning season. Many studies of these hormone levels during the breeding season had been carried out mainly in salmonids (Breton, Billard, Jalabert and Kann, 1972; Breton, Jalabert, Fostier and Billard 1975; Crim, Meyer and Donaldson, 1973; Crim, Watts and Evans, 1975; Billard et al., 1978; Bromage and Cumaranatunga, 1988; see Figure 1.2b), carp (Billard et al., 1978; Santos et al., 1986) and goldfish (Kobayashi et al., 1986; 1988; 1989; see Figure 1.2a). The GtH levels in teleost blood slowly increase during vitellogenesis and rise sharply around the time of ovulation. Levels of E<sub>2</sub> also increase and peak during vitellogenesis but they subsequently drop prior to spawning and then remain at low levels until the next spawning phase (Lambert et al., 1978; Scott et al., 1983; Nagahama, 1983; Kagawa et al., 1983; Ueda, Young, Crim, Kambegawa and Nagahama, 1984).

Progesterone and T were found at low levels during the vitellogenic phases with peaks at oocyte maturation and ovulation. For example, in goldfish, T levels increased and peaked at ovulation (Kobayashi et al., 1986; 1988; 1989) and in rainbow trout, *Onchorynchus mykiss*, these levels increased and peaked at 8 days before ovulation and then decreased thereafter (Scott et al., 1983). The levels of



17 $\alpha$ ,20 $\beta$ -P which showed a similar trend as GtH, were found at low levels during vitellogenesis and then dramatically increased and peaked sharply just prior to ovulation (Scott, et al., 1983).

The hormonal profiles of multiple spawners such as tilapias (*O. mossambicus*) are not as well defined. The profiles of T and E, during spawning cycle are unclear and unlike salmonids, the profiles can show several peaks of these hormones prior to spawning (Smith and Haley, 1988).

## 6.2 OBJECTIVES

This study attempted to elucidate the ovarian and hormonal patterns in *O. niloticus* females which were robbed of their clutch after spawning (non-mouthbrooding females).

A trial was conducted with a group of non-mouthbrooding females. The ovarian structures, hormonal profiles, total egg numbers and fertilisation rates were investigated over two spawning cycles.

In addition, the effects of stress caused by bleeding females twice a week throughout their spawning cycles, on hormonal profiles and egg quality of individual females were examined.



## 6.3 METHODS

### 6.3.1 Definitions of Terms Used in the Present Study

#### 6.3.1.1 Mouthbrooding and non-mouthbrooding females

Females that were allowed to incubate their eggs in their mouths, were termed as "mouthbrooding females". Whereas those females from which their clutches were removed and artificially incubated were termed as "non-mouthbrooding females".

#### 6.3.1.2 Spawning time and spawning cycle

The term "spawning time" was used to describe the time of oviposition (Day 0). Days 1, 5 and 10 were defined as the period (days) after each spawning time (see Figure 6.1).

The term "interspawning interval" (ISI) or "spawning cycle" referred to the period between spawning times (see Figure 6.1).



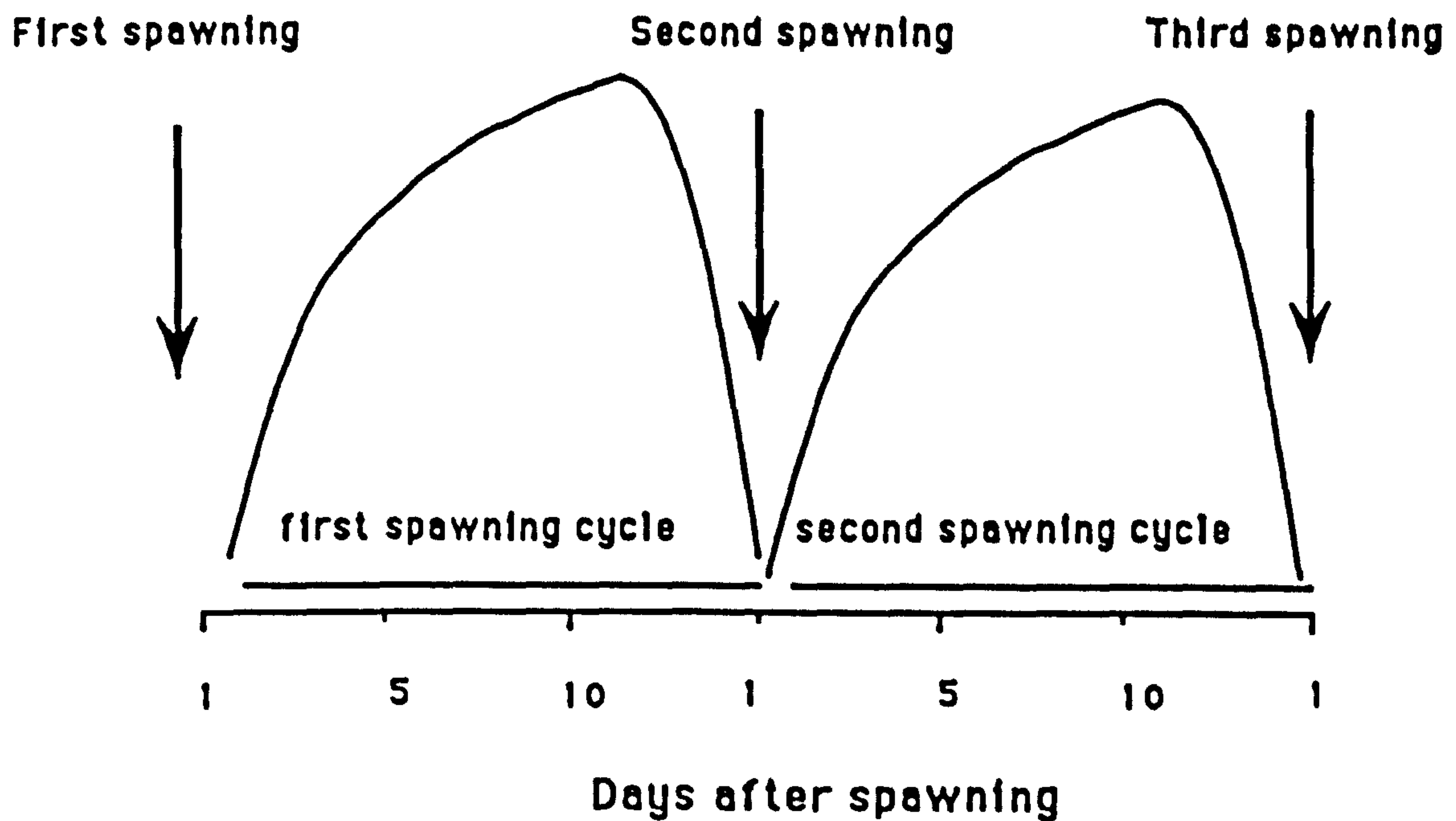


Figure 6.1: Criteria used to define the spawning history of non-mouthbrooder *O. niloticus* females in the present study. The "spawning time" indicated the time of oviposition. The "spawning cycle" indicated the interspawning interval between two spawning times.



### 6.3.2 Ovarian Recrudescence, Hormonal Profiles and Spawning Cycles of Post-Spawed Non-Mouthbrooding Females

*Oreochromis niloticus* broodstock were obtained from the common pool of mature fish reared for section 5.3.2. The fish were about 1 year old and weighed 85-220g ( $151.4 \pm 11.2$ g;  $n = 42$ ). Broodstock were hand-sexed and the males and the females stocked separately in 1 m<sup>2</sup> fibreglass tanks at a density of 20 - 30 fish/m<sup>2</sup>. Ripe females for this study were selected by their swollen and soft bellies and large, protruding red or pink genital papillae. Eight to ten selected females were then transferred into a 2 m diameter spawning tank with 3 males (Plate 6.1) and allowed to spawn naturally at a temperature of  $27 \pm 1^{\circ}\text{C}$ , with a 12L:12D photoregime.

The fish, which were fed twice a day throughout the experiment on a commercial trout pellets containing 40% protein, were observed daily for the presence of mouthbrooding females. The mouthbrooding females were gently transferred into a bucket to release the eggs. The fish were identified with numbered tags and returned to the spawning tanks. Spawning day (Day 0), egg number and fertilisation rate (eyed eggs at day 2) of each clutch were recorded.



Six females were sacrificed at 1, 5 and 10 days after their first, second and third spawnings (Figure 6.1). The fish were anaesthetized in 2-phenoxy ethanol (section 2.1.2), bled and sacrificed in an overdose of anaesthetic (2-phenoxy ethanol). Body length, weight and liver weight were recorded and the ovaries dissected, fixed and processed for histological (section 2.2.1) and electron microscopical (section 2.2.2) studies. Four to six sections from each ovarian sample were cut and stained with polychrome stain as described in section 2.2.1.2b. Ovarian stages were classified according to the details in chapter 3 (Table 3.1). Ten to fifteen separated fields were examined and the volume fraction of the different stages of oocytes estimated (section 2.3).

The serum samples were analyzed for total calcium, testosterone and oestradiol-17 $\beta$  according to sections 2.4 and 2.5, respectively.

### 6.3.3 Effect of Bi-weekly Bleeding Stress on spawning Cycles, Egg Quality and Hormone Profiles of the Individual Females (Experiment 2)

Non-mouthbrooder females (*O. niloticus*) were obtained and maintained under the same conditions as described in experiment 1 (section 6.3.2).



At the first detected spawning (first spawning), the eggs were removed and the non-mouthbrooding females were anaesthetized and bled (0.3ml) twice a week. The spawning history of eight such individual females were followed for between two to five spawning cycles. Total numbers and quality of eggs from individual females were recorded. Serum samples from the individual females were analyzed for total calcium, oestradiol-17 $\beta$  and testosterone according to the details described in sections 2.4 and 2.5, respectively.





Plate 6.1: Spawning tanks used for *O. niloticus* in the present study. The fish were stocked at a ratio of 3 females : 1 male and allowed to spawn naturally.



## 6.4 RESULT

### 6.4.1 Ovarian Recrudescence, Hormonal Profiles and Spawning Cycles of Post-Spawned Non-Mouthbrooding Females (Experiment 1)

#### 6.4.1.1 Size of fish

The mean length, weight and HSI of the 42, one year old females used in this study, were 20.4 cm, 150.2 g and 2.6%, respectively, and the differences within each of these parameters throughout the present study were not significant ( $P>0.05$ ) (Table 6.1).

#### 6.4.1.2 Recrudescence of oocytes during spawning cycles

##### a. Gonadosomatic index (GSI)

At day 1 after the first, second and third spawning, the mean GSIs were 2.1, 1.5 and 1.6%, respectively. There was no statistical difference ( $P>0.05$ ) between the GSI during this period. The GSIs increased from 1.5 - 2.1% on day 1 after spawning to between 2.5 - 3.0% and 3.9 - 4.5% by days 5 and 10 after spawning, respectively (Table 6.1). The GSIs at days 5 and 10 post-spawning were significantly ( $P<0.05$ ) higher than those at day 1 post-spawning.



## **b. Gonadal changes during ovarian recrudescence**

Ovaries of the females sacrificed at day 1 after spawning contained various sizes and stages of oocytes (Plates 6.2 and 6.3). The composition of these ovaries, however, varied depending on the spawning history of the females. The ovaries sacrificed after the first spawning contained an average of 24.8 and 40.8% of stage 5 and atretic oocytes, respectively, and oocyte stages 2, 3 and 4 accounted for 15.1% in the ovaries.

When the oocyte structures in day 1 post-spawned ovaries were compared at the different spawning times, the atretic and stage 5 oocyte volume fractions were found to be significantly ( $P < 0.05$ ) greater than those of stage 2, 3 and 4 oocytes (Plate 6.2; 6.3). By the second and third spawning, however, these volume fractions of stage 5 and atretic oocytes decreased from 24.8 and 40.8% to 22 and 17-18%, respectively.

By days 5 and 10 after each spawning, stage 6 oocytes occupied the greater part of the ovary when compared with other oocyte stages (Plate 6.2, 6.3). The stage 6 oocyte volume fraction increased from 0 - 15.5% on day 1 to the range of 58.1 - 60.1% and 64.7 - 71.9% by days 5 and 10 after spawning, respectively. The volume fractions of stage 6 oocytes at days 5 and 10 after spawning were not significantly different ( $P > 0.05$ ).



In contrast, the volume fraction of atretic oocytes in the ovary were dramatically reduced from 17.9 - 40.8% on day 1 to 9.9 - 21.4% and 1.0 - 7.7% on days 5 and 10 after spawning, respectively. The stage 5 oocytes also decreased significantly ( $P < 0.05$ ) from 22.2 - 24.8% on day 1 to 4.5 - 7.1% and 4.3 - 7.3%, respectively.

### **c. Ultrastructure of oocyte follicles**

Ultrastructural studies of ovaries at day 1 after spawning showed that the follicular cells of stage 5 oocytes contained little cytoplasm and consisted of a flat, small thecal layer. The thecal and granulosa layers were separated by basement membrane (Plate 6.4a,b). The granulosa cells, on the other hand, contained large nuclei with well developed mitochondria (Plate 6.4a); whereas no mitochondria were found in the thecal cells. The zona pellucida was well developed and contained numerous microvilli between the granulosa layer and the oocyte ooplasm. These microvilli were used to transfer 'material' by pinocytosis from the granulosa layer via the zona pellucida into the oocyte ooplasm (see Plate 6.6).



The morphology of the thecal and granulosa cells changed during ovarian recrudescence. Thecal cells at day 1 after spawning were small and then increased in size (i.e., cytoplasm and nuclei) by days 5 and 10 after spawning (Plate. 6.4; 6.5). The shape of the nuclei of thecal and granulosa cells at day 1 after spawning were oblong and ellipsoid; by days 5 and 10 after spawning, they changed to irregular and round shape. The length of microvilli at day 1 was longer than the length at days 5 and 10 after spawning (Figure 6.4; 6.5). Thickness of the zona pellucida increased at days 5 and 10 after spawning (Figure 6.6). The well developed mitochondria and microfilament, which appeared at ovulation were initially found in the cytoplasm of thecal cells at days 5 after spawning (Plate 6.5a).



Table 6.1: Fish size, HSI, GSI, total calcium, oestradiol-17 $\beta$  and testosterone (mean $\pm$ SE; n = 6) at different periods after spawning during the first and second spawning cycle of *O. niloticus*

| Cycle | Days after spawning | Length (cm)                     | Weight (g)                       | HSI (%)                        | GSI (%)                         | Ca <sup>2+</sup> (mg%)           | T (ng/ml)                       | E <sub>2</sub> (ng/ml)          |
|-------|---------------------|---------------------------------|----------------------------------|--------------------------------|---------------------------------|----------------------------------|---------------------------------|---------------------------------|
| 1     | 1                   | 19.1 <sup>a</sup><br>$\pm 0.84$ | 131.3 <sup>a</sup><br>$\pm 18.3$ | 2.9 <sup>a</sup><br>$\pm 0.22$ | 2.1 <sup>a</sup><br>$\pm 0.29$  | 31.6 <sup>a</sup><br>$\pm 5.36$  | 4.7 <sup>a</sup><br>$\pm 0.95$  | 3.2 <sup>a</sup><br>$\pm 0.78$  |
|       | 5                   | 19.8 <sup>a</sup><br>$\pm 0.73$ | 134.0 <sup>a</sup><br>$\pm 12.9$ | 3.1 <sup>a</sup><br>$\pm 0.32$ | 3.0 <sup>b</sup><br>$\pm 0.11$  | 32.6 <sup>a</sup><br>$\pm 2.22$  | 41.2 <sup>b</sup><br>$\pm 11.8$ | 33.6 <sup>b</sup><br>$\pm 6.07$ |
|       | 10                  | 19.9 <sup>a</sup><br>$\pm 0.73$ | 138.4 <sup>a</sup><br>$\pm 9.52$ | 3.0 <sup>a</sup><br>$\pm 0.33$ | 3.9 <sup>b</sup><br>$\pm 0.58$  | 45.0 <sup>a</sup><br>$\pm 9.10$  | 65.1 <sup>b</sup><br>$\pm 17.8$ | 23.8 <sup>b</sup><br>$\pm 5.79$ |
| 2     | 1                   | 20.5 <sup>a</sup><br>$\pm 0.77$ | 152.6 <sup>a</sup><br>$\pm 17.4$ | 2.4 <sup>a</sup><br>$\pm 0.23$ | 1.5 <sup>a</sup><br>$\pm 0.14$  | 38.8 <sup>a</sup><br>$\pm 4.08$  | 4.7 <sup>a</sup><br>$\pm 0.87$  | 1.8 <sup>a</sup><br>$\pm 0.80$  |
|       | 5                   | 20.6 <sup>a</sup><br>$\pm 0.89$ | 160.2 <sup>a</sup><br>$\pm 21.5$ | 2.5 <sup>a</sup><br>$\pm 0.34$ | 2.5 <sup>b</sup><br>$\pm 0.32$  | 28.0 <sup>a</sup><br>$\pm 1.39$  | 30.9 <sup>b</sup><br>$\pm 3.09$ | 37.8 <sup>b</sup><br>$\pm 4.84$ |
|       | 10                  | 21.0 <sup>a</sup><br>$\pm 0.93$ | 163.3 <sup>a</sup><br>$\pm 20.7$ | 2.3 <sup>a</sup><br>$\pm 0.22$ | 4.5 <sup>b</sup><br>$\pm 0.48$  | 40.5 <sup>a</sup><br>$\pm 6.03$  | 44.1 <sup>b</sup><br>$\pm 7.47$ | 26.1 <sup>b</sup><br>$\pm 1.15$ |
| 3     | 1                   | 21.2 <sup>a</sup><br>$\pm 0.83$ | 171.6 <sup>a</sup><br>$\pm 19.6$ | 2.4 <sup>a</sup><br>$\pm 0.15$ | 1.57 <sup>a</sup><br>$\pm 0.13$ | 34.33 <sup>a</sup><br>$\pm 2.31$ | 11.14 <sup>a</sup><br>$\pm 5.6$ | 3.63 <sup>a</sup><br>$\pm 0.96$ |

Means ( $\pm$ SE) with same superscripts within columns indicate no significant (P>0.05) differences.



Table 6.2: Ovarian volume fractions of the different oocyte stages at various times after spawning and spawning cycles of *O.niloticus*

| Cycle | Day after spawning | % Ovarian volume fraction (mean $\pm$ SE) <sup>1</sup> |                                |                                |                                 |                                 |                                 | Post-F <sup>2</sup>             |
|-------|--------------------|--|--------------------------------|--------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
|       |                    | stage 2  | stage 3                        | stage 4                        | stage 5                         | stage 6                         | Atresia                         |                                 |
| 1     | 1                  | 5.6 <sup>a</sup><br>$\pm 0.13$                         | 3.9 <sup>a</sup><br>$\pm 1.15$ | 5.6 <sup>a</sup><br>$\pm 1.28$ | 24.8 <sup>b</sup><br>$\pm 6.26$ | 0.0 <sup>a</sup>                | 40.8 <sup>b</sup><br>$\pm 4.52$ | 16.2 <sup>b</sup><br>$\pm 2.69$ |
|       | 5                  | 3.2 <sup>a</sup><br>$\pm 0.72$                         | 3.6 <sup>a</sup><br>$\pm 0.54$ | 5.1 <sup>a</sup><br>$\pm 0.99$ | 4.5 <sup>a</sup><br>$\pm 1.69$  | 60.1 <sup>b</sup><br>$\pm 5.83$ | 21.4 <sup>b</sup><br>$\pm 8.84$ | 1.7 <sup>a</sup><br>$\pm 0.99$  |
|       | 10                 | 4.5 <sup>a</sup><br>$\pm 0.92$                         | 3.8 <sup>a</sup><br>$\pm 1.06$ | 5.2 <sup>a</sup><br>$\pm 1.76$ | 4.3 <sup>a</sup><br>$\pm 1.85$  | 71.9 <sup>b</sup><br>$\pm 3.39$ | 7.7 <sup>a</sup><br>$\pm 7.11$  | 0.8 <sup>a</sup><br>$\pm 0.78$  |
| 2     | 1                  | 5.6 <sup>a</sup><br>$\pm 0.84$                         | 4.2 <sup>a</sup><br>$\pm 0.96$ | 2.1 <sup>a</sup><br>$\pm 1.25$ | 22.2 <sup>b</sup><br>$\pm 4.47$ | 15.3 <sup>b</sup><br>$\pm 8.92$ | 17.9 <sup>b</sup><br>$\pm 7.62$ | 18.8 <sup>b</sup><br>$\pm 2.33$ |
|       | 5                  | 5.4 <sup>a</sup><br>$\pm 0.30$                         | 4.6 <sup>a</sup><br>$\pm 1.03$ | 8.1 <sup>a</sup><br>$\pm 1.41$ | 7.1 <sup>a</sup><br>$\pm 3.21$  | 58.1 <sup>b</sup><br>$\pm 0.64$ | 9.9 <sup>a</sup><br>$\pm 0.98$  | 9.5 <sup>a</sup><br>$\pm 2.59$  |
|       | 10                 | 6.6 <sup>a</sup><br>$\pm 0.42$                         | 5.0 <sup>a</sup><br>$\pm 1.53$ | 5.8 <sup>a</sup><br>$\pm 2.88$ | 7.3 <sup>a</sup><br>$\pm 0.76$  | 64.7 <sup>b</sup><br>$\pm 1.34$ | 1.0 <sup>a</sup><br>$\pm 0.96$  | 0.0 <sup>a</sup>                |
| 3     | 1                  | 5.3 <sup>a</sup><br>$\pm 0.59$                         | 4.4 <sup>a</sup><br>$\pm 0.89$ | 6.5 <sup>a</sup><br>$\pm 1.05$ | 22.7 <sup>b</sup><br>$\pm 2.94$ | 4.9 <sup>a</sup><br>$\pm 4.87$  | 16.6 <sup>b</sup><br>$\pm 6.04$ | 27.4 <sup>b</sup><br>$\pm 5.77$ |

Means ( $\pm$ SE) with same superscripts within columns indicate no significant (P>0.05) differences.

<sup>1</sup> n = 4 - 5

<sup>2</sup> = post-ovulatory follicle



Plate 6.2 Changes in ovarian development of *O.niloticus* females during the first spawning cycle.

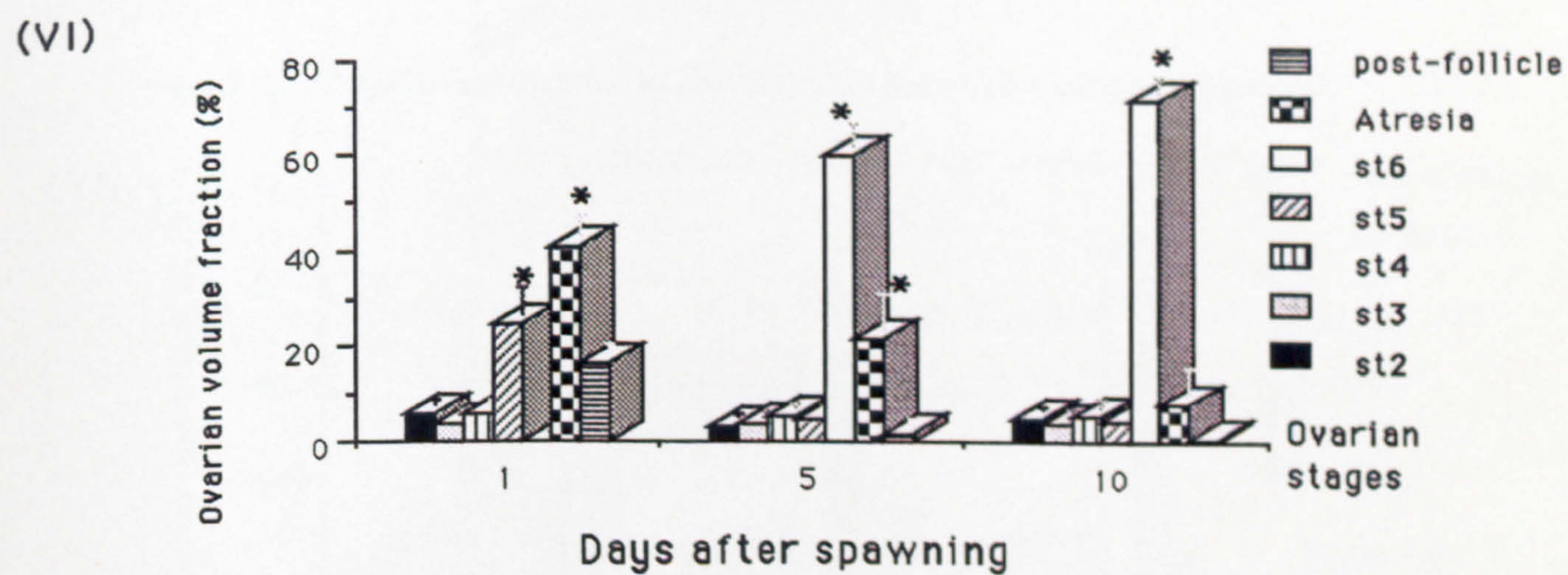
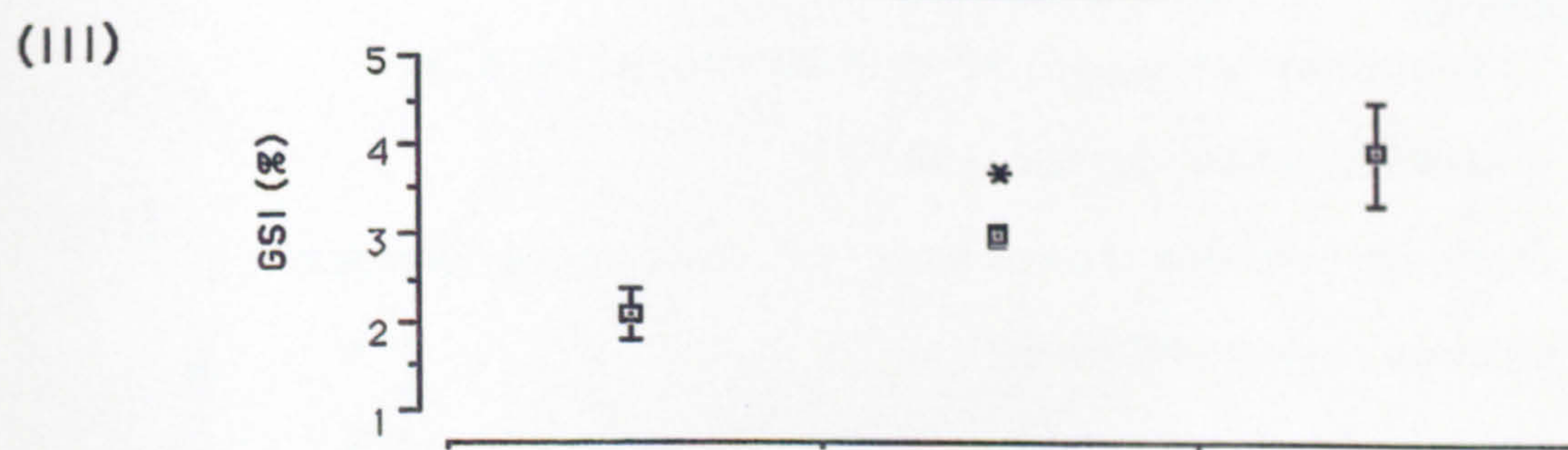
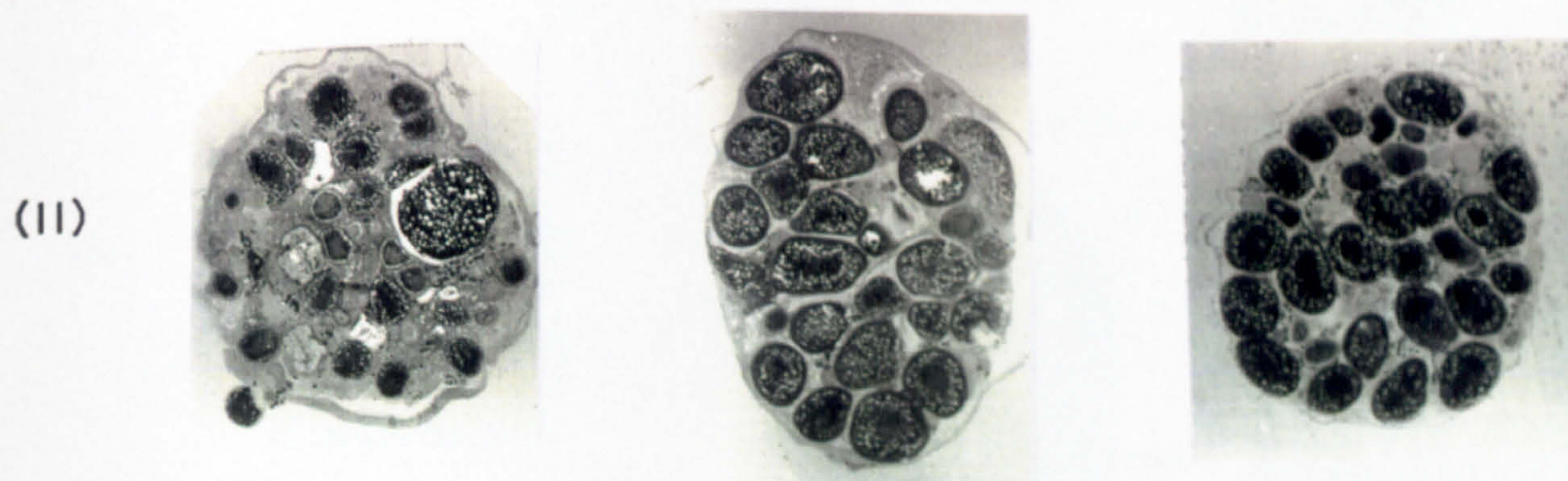
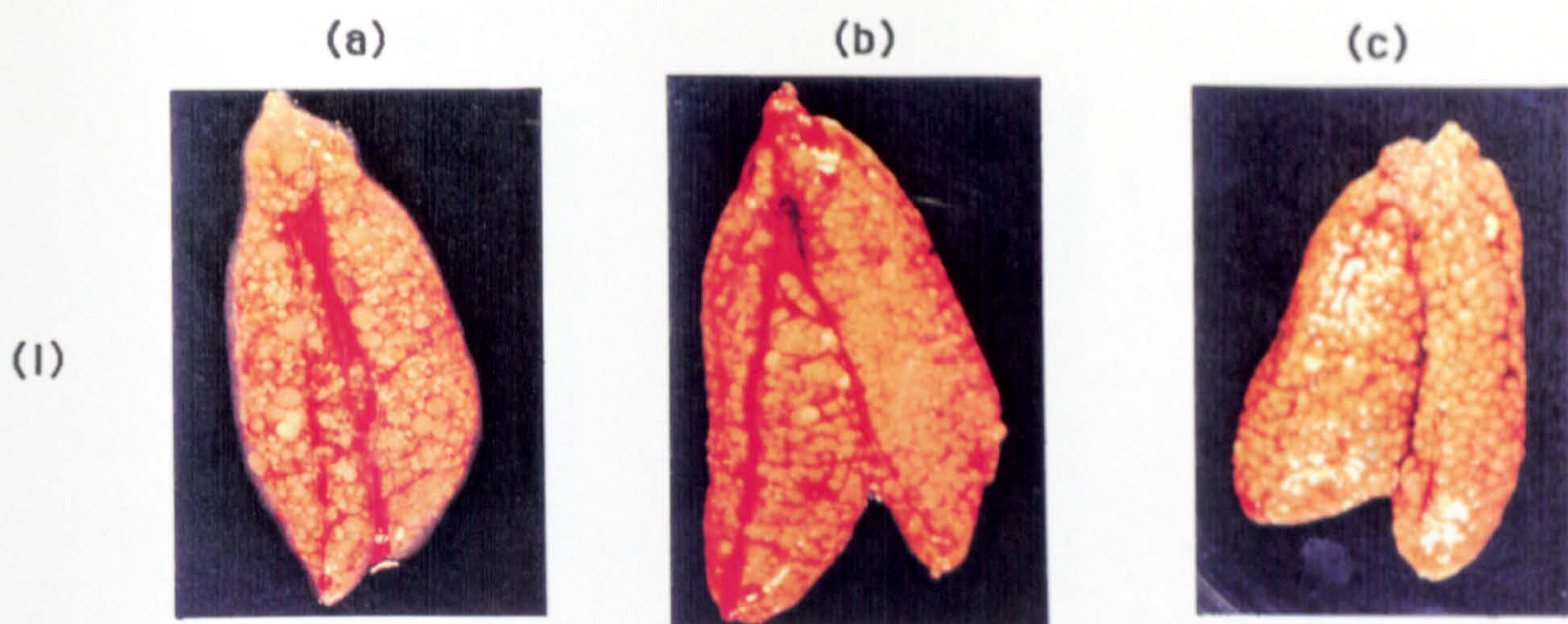
- (a) Day 1 after spawning
- (b) Day 5 after spawning
- (c) Day 10 after spawning

- (I) Whole ovaries (x 1.1)
- (II) Transverse section of the ovaries (x 4.8)
- (III) Gonadosomatic index (GSI)
- (VI) Ovarian volume fractions of different oocyte stages (means $\pm$ SE;n=6) in the ovaries

(III) \* Indicates significantly ( $P < 0.05$ ) higher GSI than the others between different periods.

(VI) \* Indicates significantly ( $P < 0.05$ ) higher values than the others at the same period.





Cycle 1

Plate 6.2



Plate 6.3 Changes in ovarian development of *O.niloticus* females during the second spawning cycle.

- (a) Day 1 after spawning
- (b) Day 5 after spawning
- (c) Day 10 after spawning

- (I) Whole ovaries (x 1.1)
- (II) Transverse section of the ovaries (x 4.8)
- (III) Gonadosomatic index (GSI)
- (VI) Ovarian volume fractions of different oocyte stages (mean $\pm$ SE;n=6)

(III) \* Indicates significantly ( $P < 0.05$ ) higher GSI than the others between different periods.

(VI) \* Indicates significantly ( $P < 0.05$ ) higher values than the others at the same period.



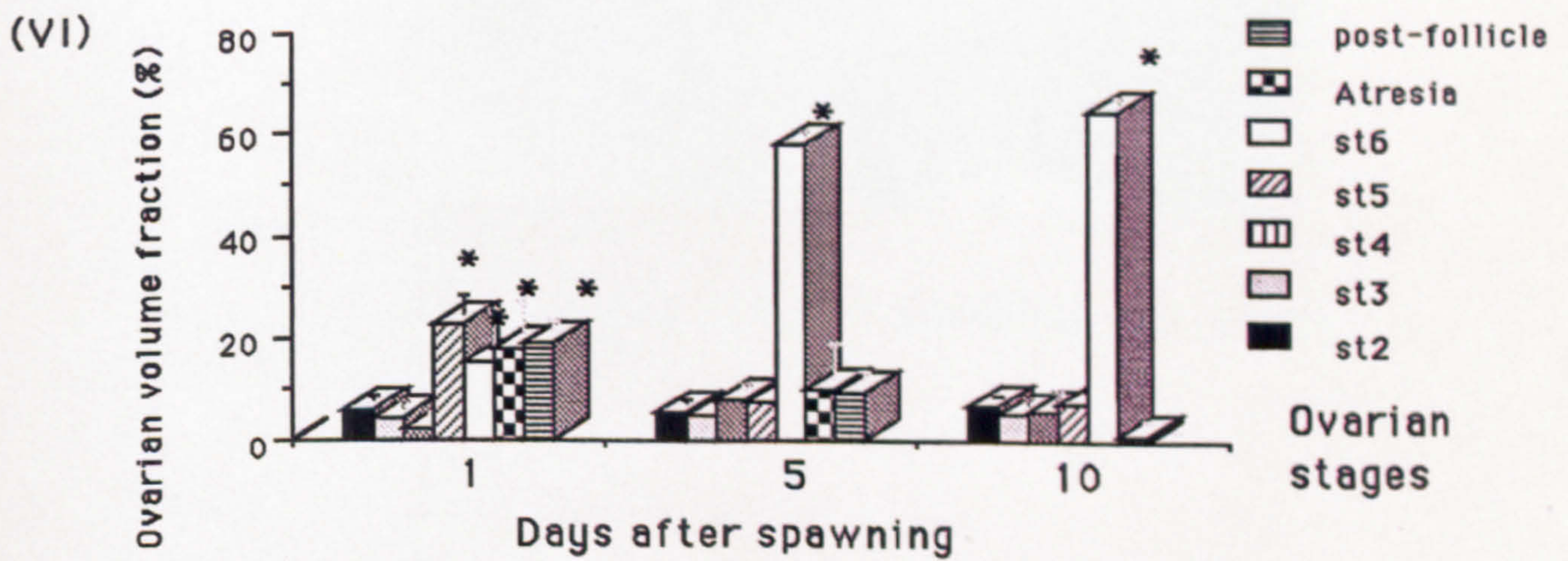
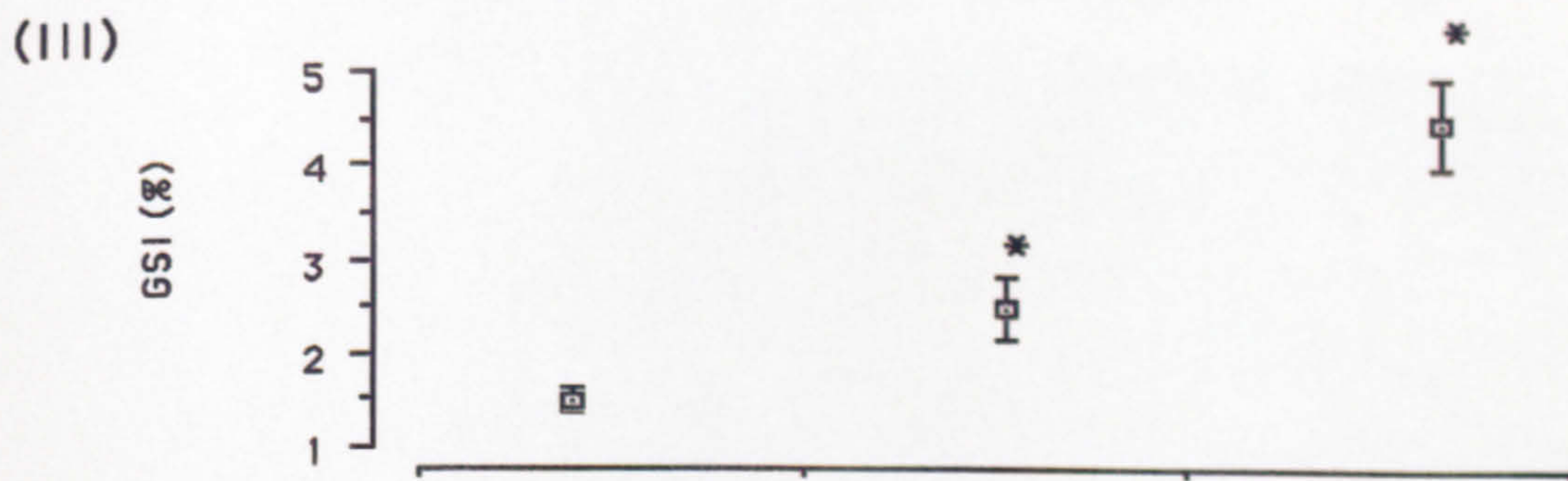
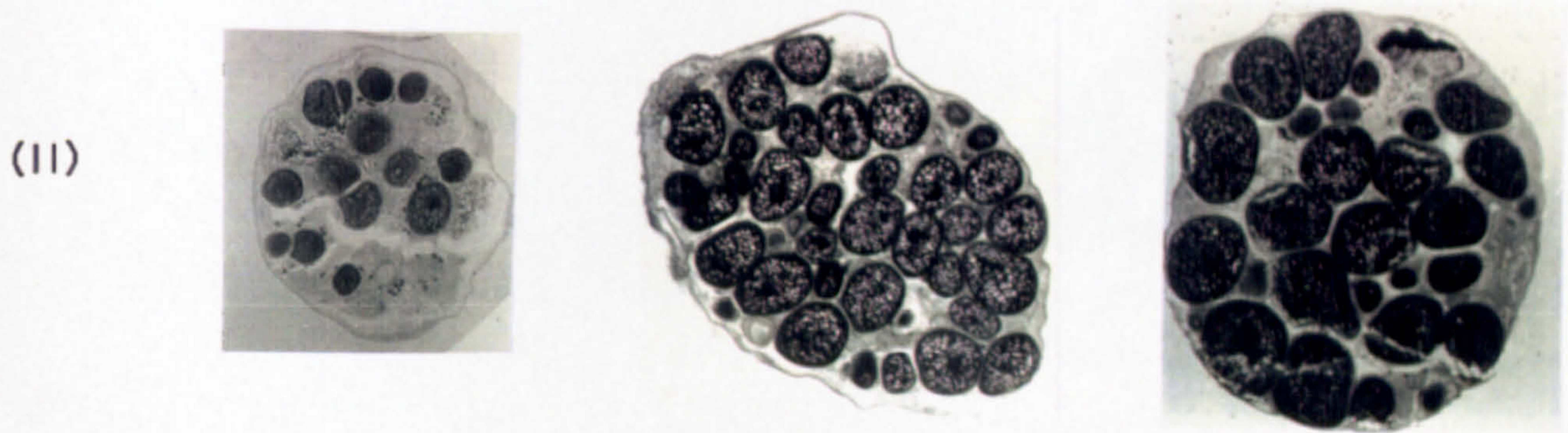




Plate 6.4 Electron micrographs of mature follicles from *O.niloticus* ovaries at different periods of the reproductive cycles.

(6.4a) Day 1 after spawning (x 7,500)

(6.4b) Day 5 after spawning (x 7,500)

TH = thecal cells

GR = nucleus of granulosa cells

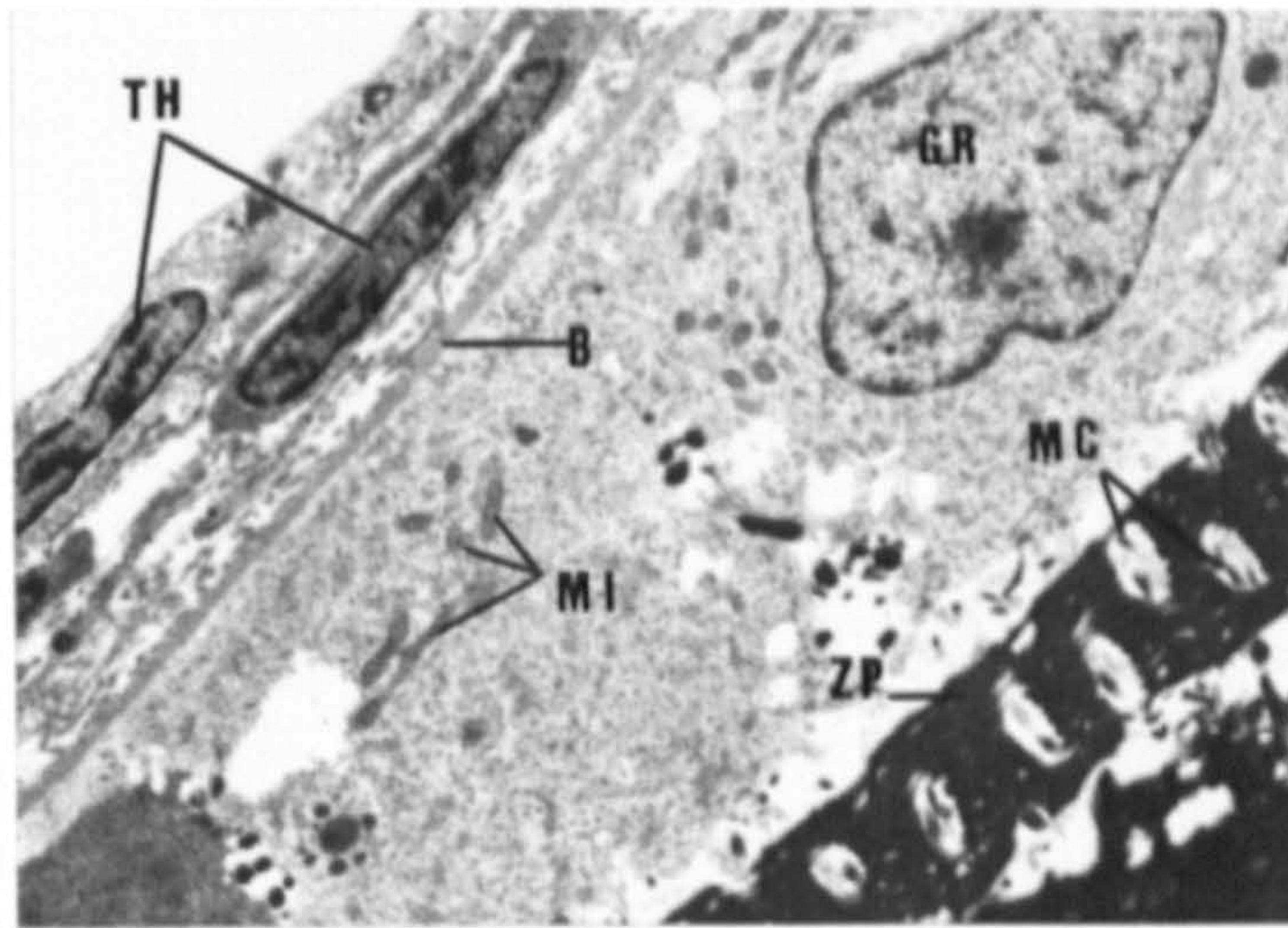
B = basement membrane

MI = mitochondria

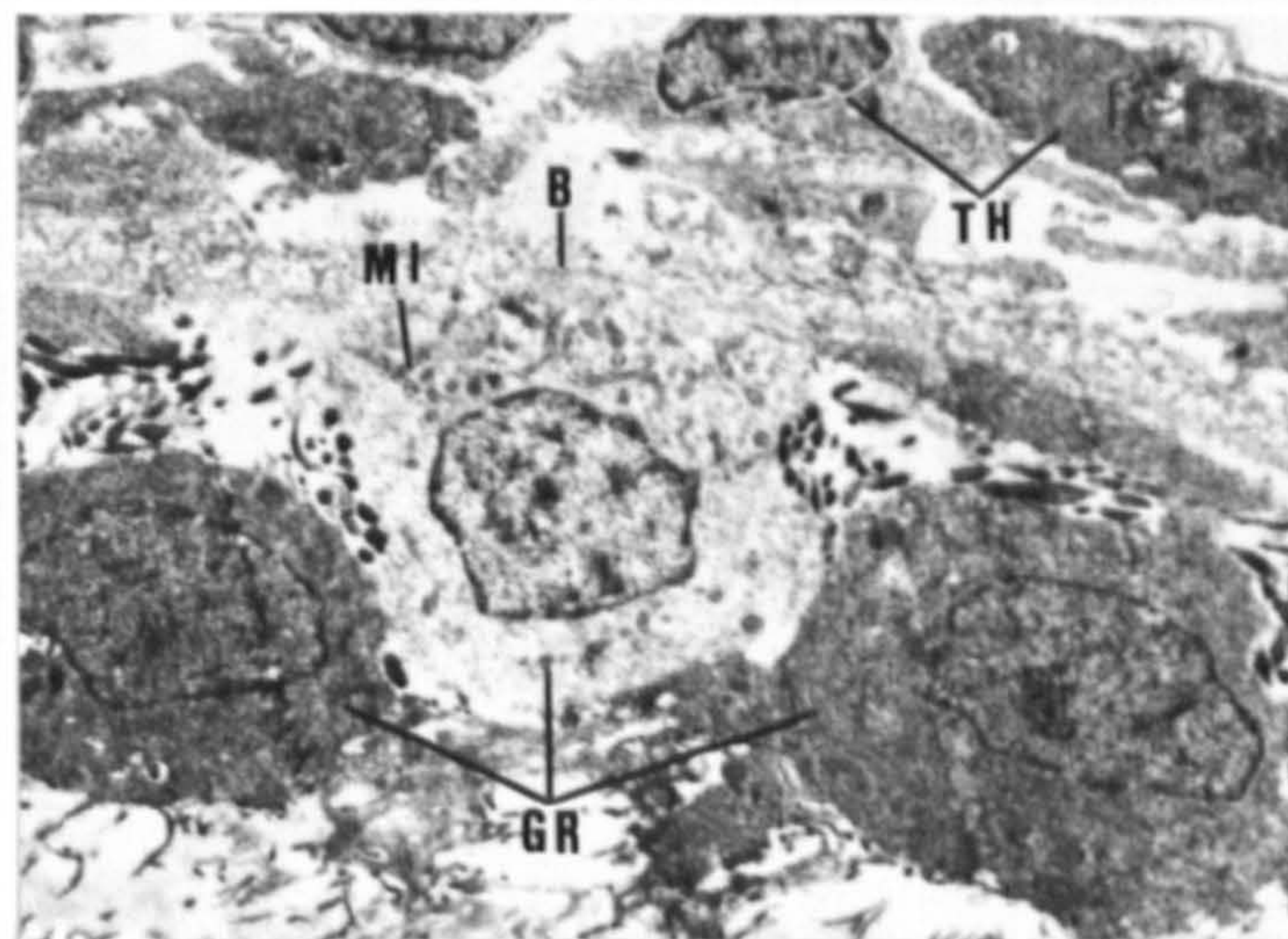
MC = microvilli

ZP = zona pellucida





(Plate 6.4a) Day 1 after spawning (x 7,500)



(Plate 6.4b) Day 5 after spawning (x 7,500)



Plate 6.5 Electron micrographs of maturing follicles of *O. niloticus* at different periods after spawning.

(6.5a) Day 5 after spawning

(6.5b) Day 10 after spawning

TH = thecal cells

GR = granulosa cells

B = basement membrane

MI = mitochondria

MC = microvilli

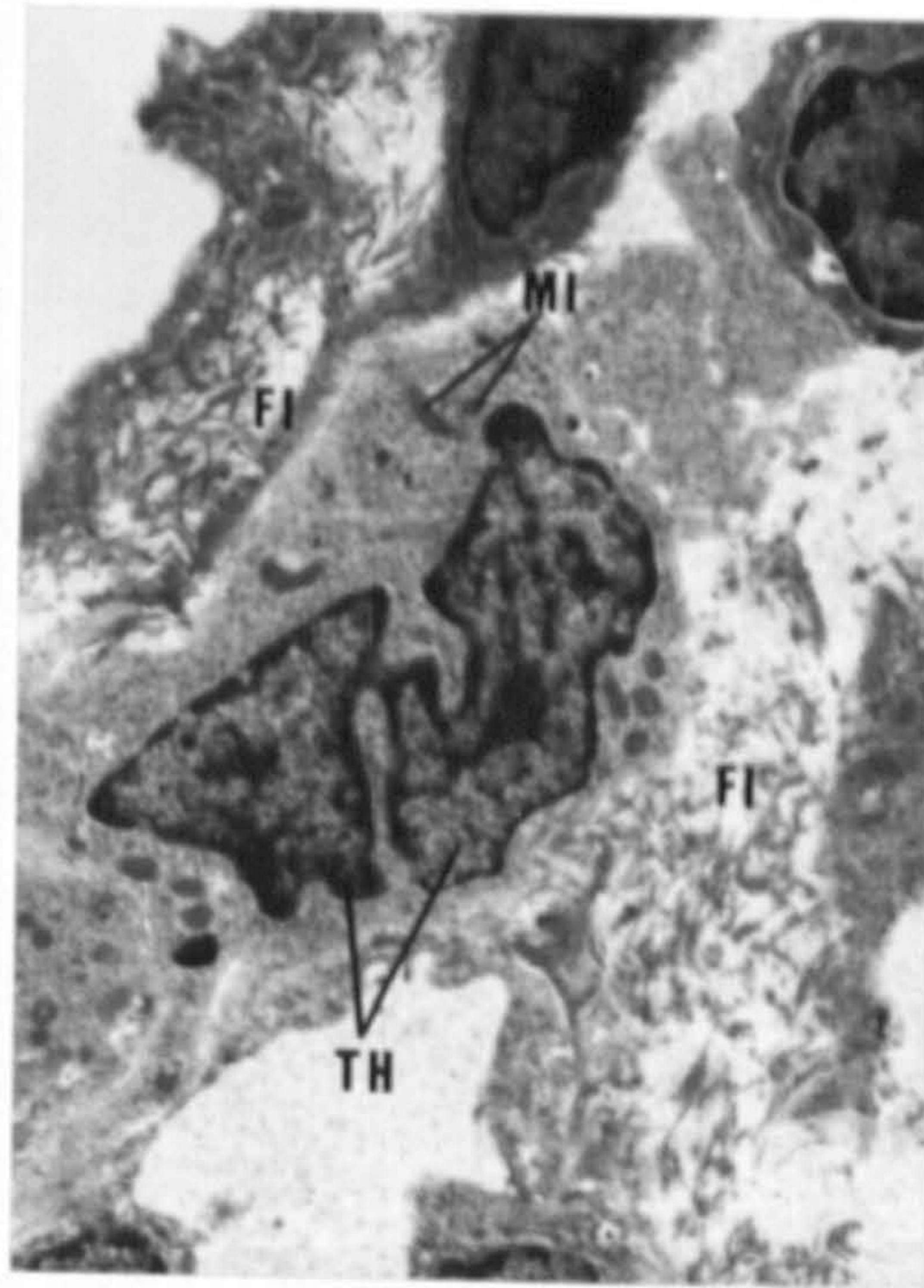
FI = microfilament

ZP = zona pellucida

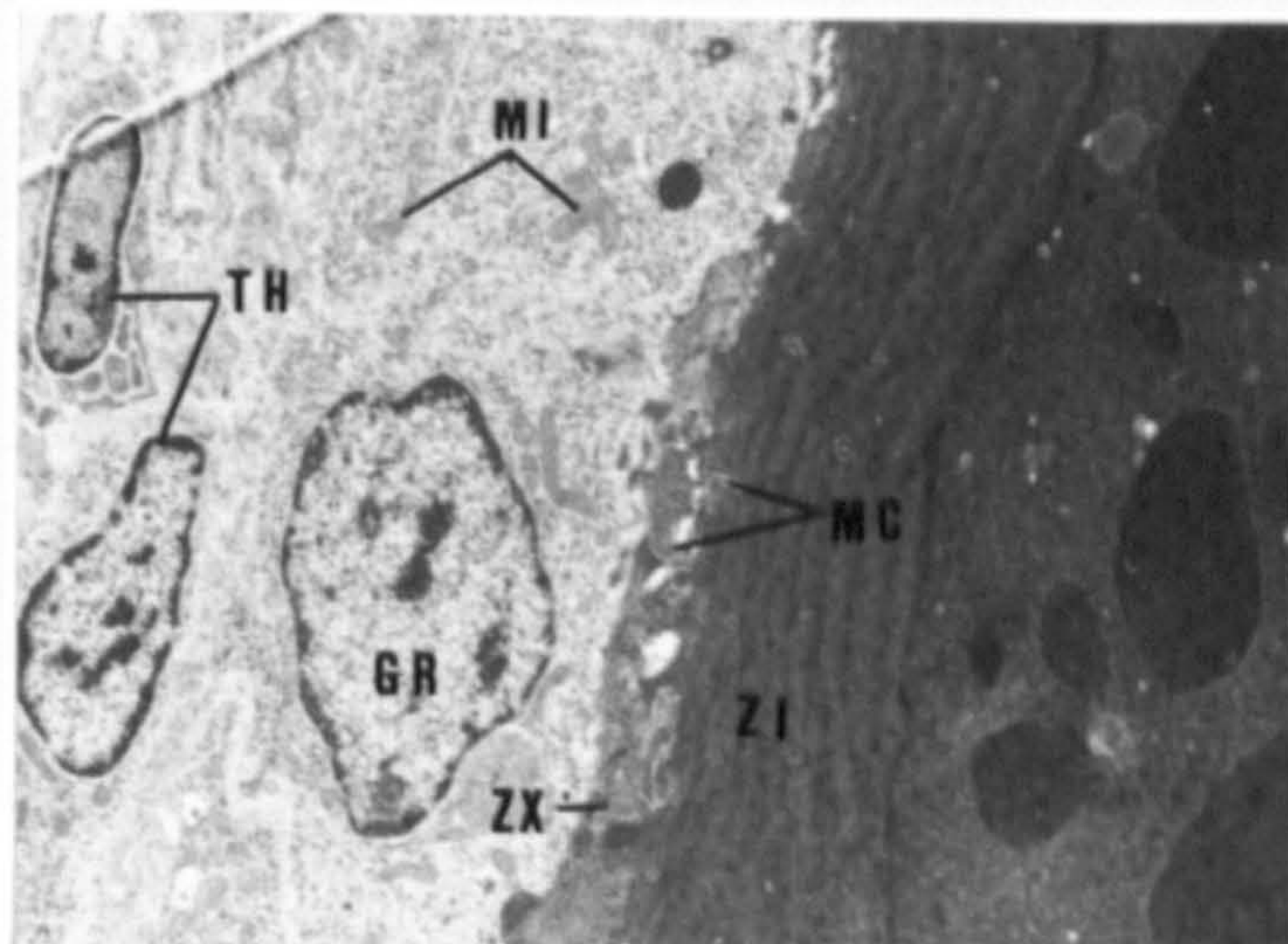
ZI = zona pellucida interna

ZX = zona pellucida externa





(Plate 6.5a) Day 5 after spawning (x 7,500)



(Plate 6.5b) day 10 after spawning (x7,500)



Plate 6.6 Electron micrographs showing pinocytosis of material passing via microvilli and zona pellucida of *O.niloticus* follicles.

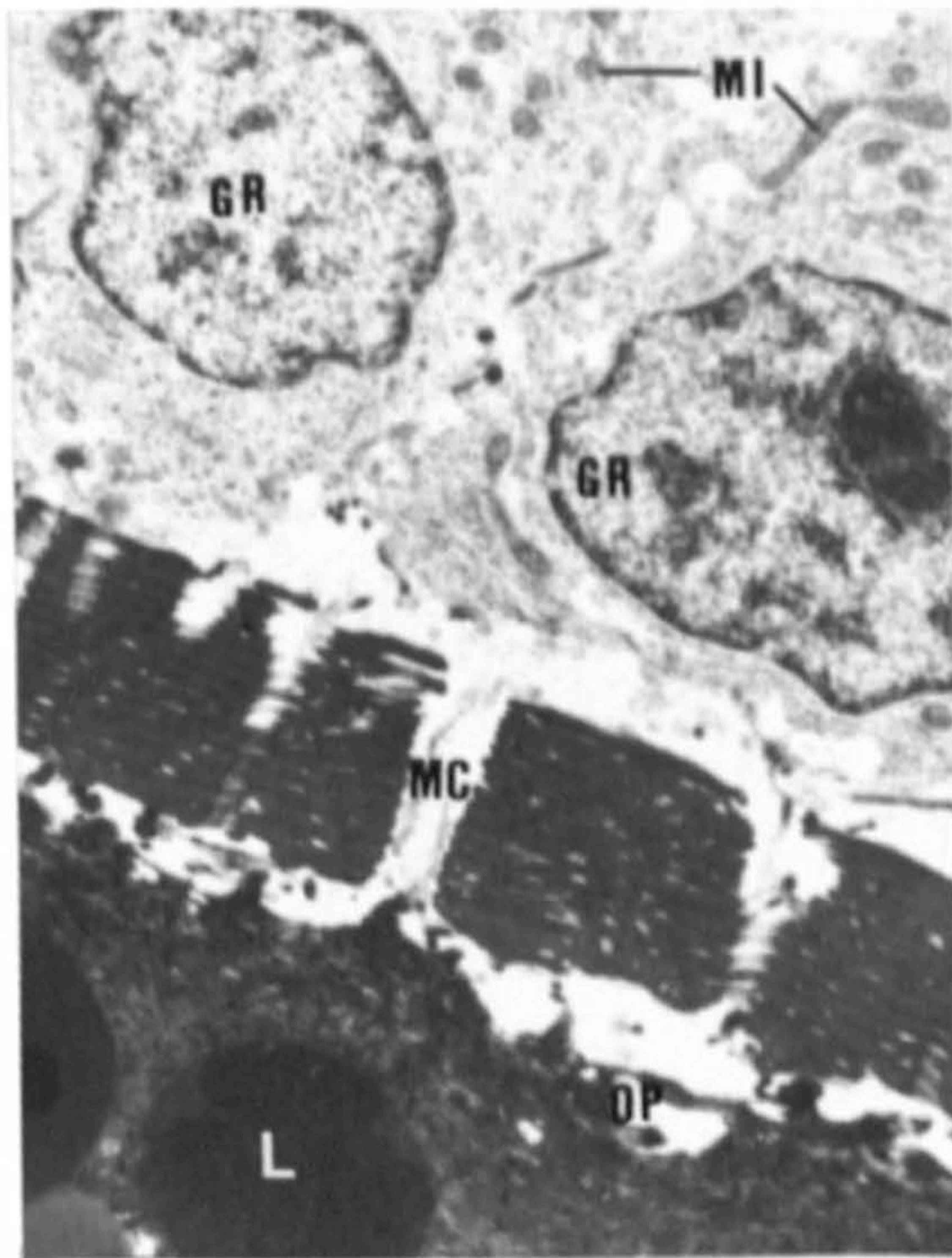
(6.6a) Day 1 after spawning (x 9,800) Note, channels of microvilli.

(6.6b) Day 10 after spawning (x 22,000) Note, material pass through zona pellucida.

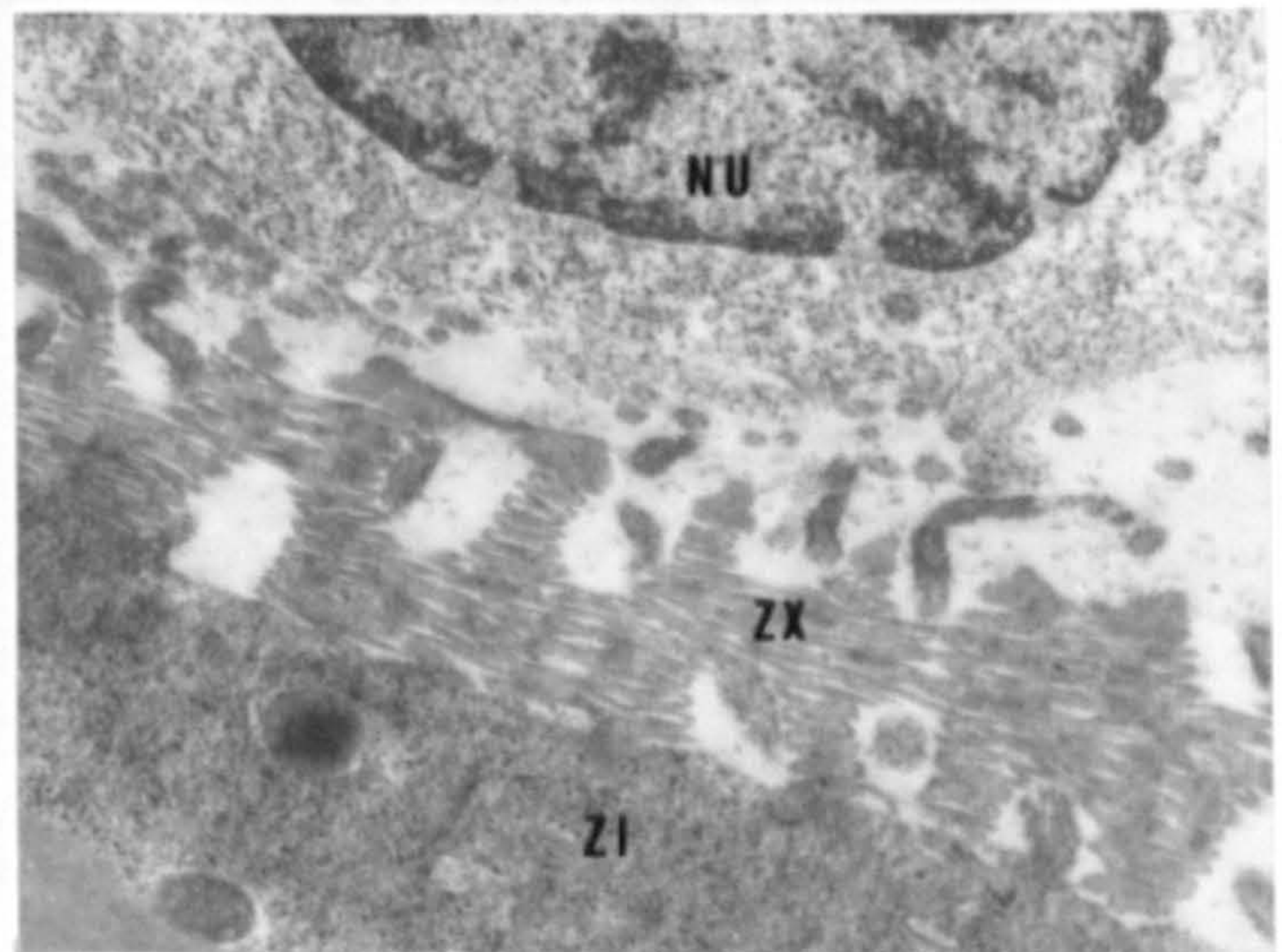
(6.6c) Day 10 after spawning (X 28,000) Note, structure of zona pellucida externa.

GR = granulosa cell  
MI = mitochondria  
ZX = zona pellucida externa  
ZI = zona pellucida interna  
OP = ooplasm  
NU = nucleus  
L = lipids  
MC = microvilli

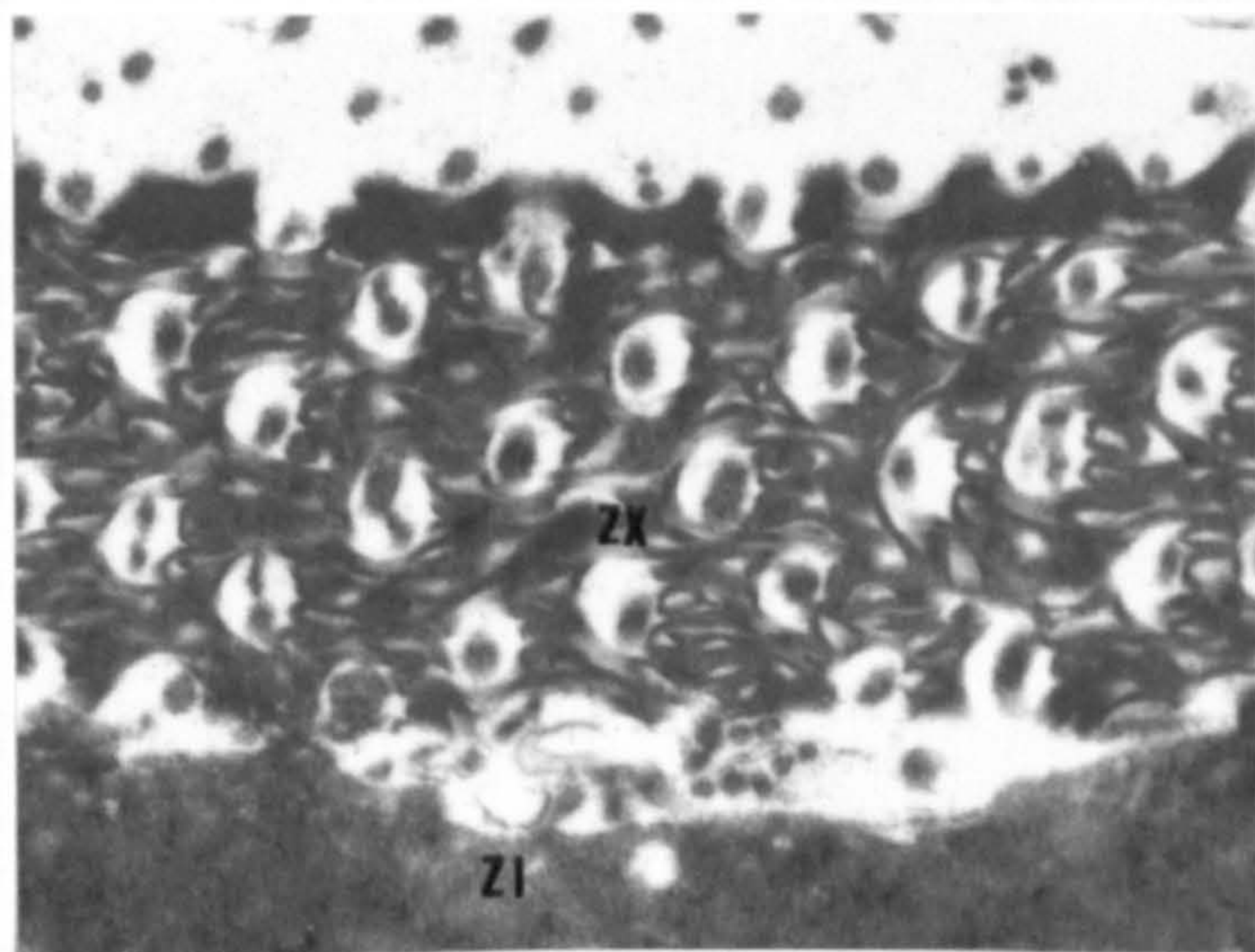




(Plate 6.6a) Day one post-spawning (x9,800)



(Plate 6.6b) Day ten post-spawning (x22,000)



(Plate 6.6c) Day ten post-spawning (x 28,000)



Figure 6.2 Comparison of reproductive parameters of non-mouthbrooding *O. niloticus* females at different periods after spawning:

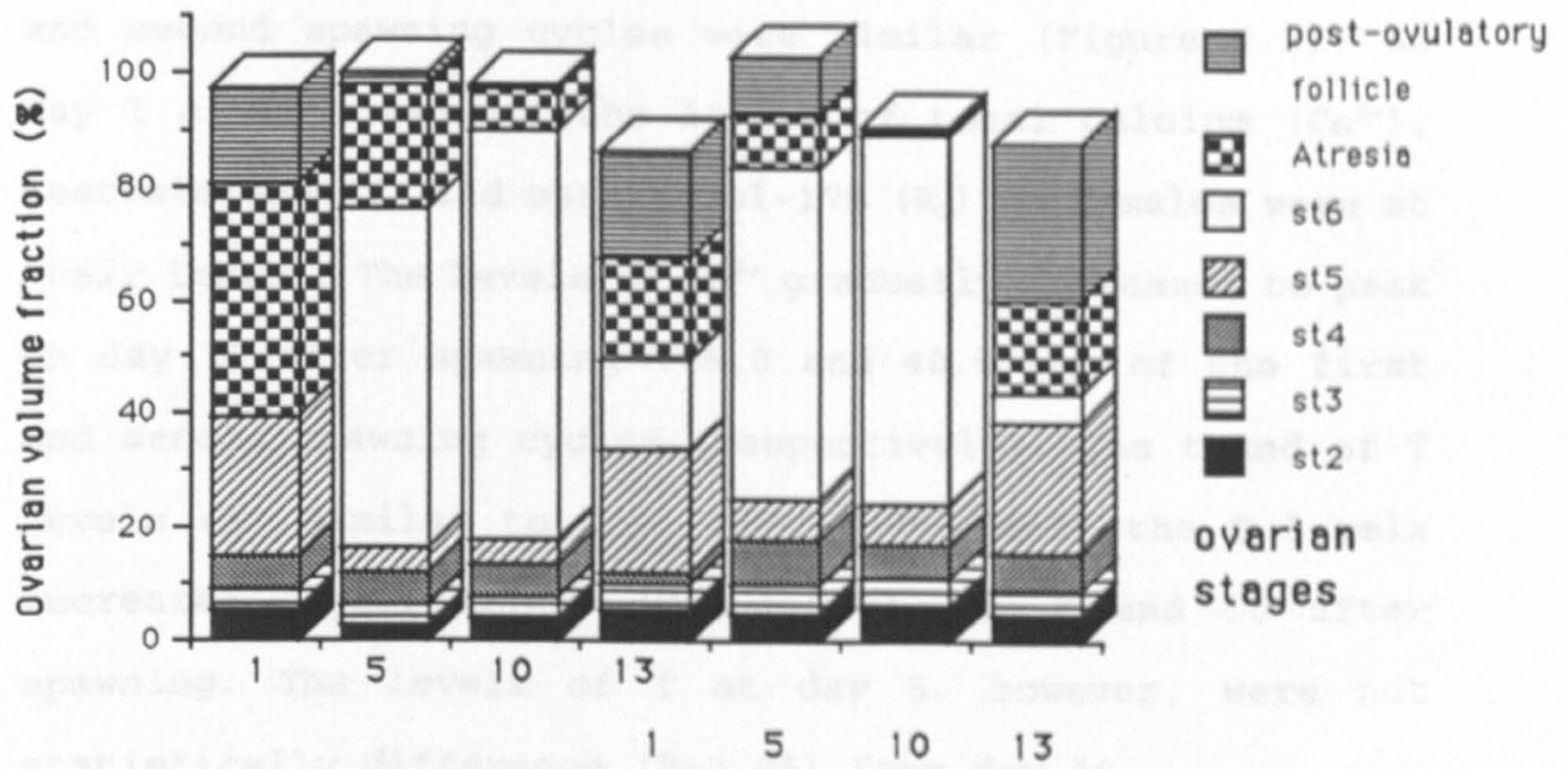
(a) Average oocyte volume fractions of different stages in the ovaries

(b) Average levels of gonadotropic index (GSI), total calcium ( $\text{Ca}^{2+}$ ), testosterone (T) and oestradiol-17 $\beta$  ( $\text{E}_2$ )

Data from Table 6.1; 6.2; during the first and second spawning cycles



(a)



(b)

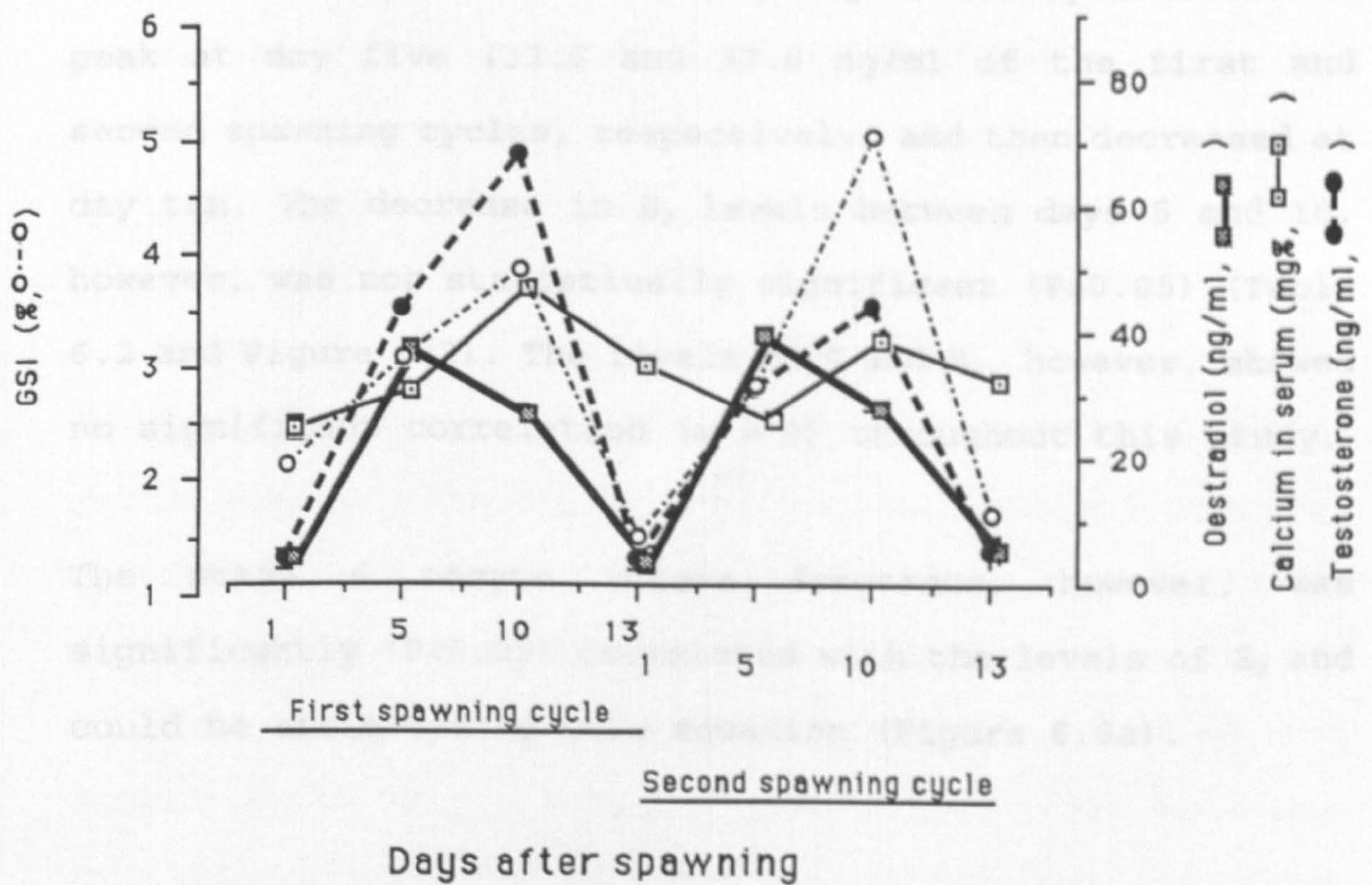


Figure 6.2



#### 6.4.1.3 Hormonal profiles during ovarian recruitment

The hormonal profiles and ovarian patterns during the first and second spawning cycles were similar (Figure 6.2). At day 1 after spawning, the levels of total calcium ( $\text{Ca}^{2+}$ ), testosterone (T) and oestradiol-17 $\beta$  ( $\text{E}_2$ ) in females were at their lowest. The levels of  $\text{Ca}^{2+}$  gradually increased to peak on day 10 after spawning (45.0 and 40.5 mg% of the first and second spawning cycles, respectively). The trend of T levels was similar to that of  $\text{Ca}^{2+}$  but only the T levels increased significantly ( $P < 0.05$ ) at day 5 and 10 after spawning. The levels of T at day 5, however, were not statistically difference ( $P > 0.05$ ) from day 10.

In contrast,  $\text{E}_2$  levels increased significantly ( $P < 0.05$ ) to peak at day five (33.6 and 37.8 ng/ml of the first and second spawning cycles, respectively) and then decreased at day ten. The decrease in  $\text{E}_2$  levels between days 5 and 10, however, was not statistically significant ( $P > 0.05$ ) (Table 6.2 and Figure 6.2). The levels of T and  $\text{E}_2$ , however, showed no significant correlation ( $r = 0$ ) throughout this study.

The stage 6 oocyte volume fractions, however, was significantly ( $P < 0.05$ ) correlated with the levels of  $\text{E}_2$  and could be estimated by this equation (Figure 6.6a).



$\%$  stage 6 =  $45.46 \log E_2 - 8.9$      $r^2 = 0.79$ ;  $df = 23$   
when:

stage 6        = volume fraction of stage 6 oocytes ( $\%$ )

$E_2$             = oestradiol-17 $\beta$  (ng/ml)

The levels of T and  $E_2$  had increased significantly ( $P < 0.05$ ) by day 5 after spawning and this coincided with an increase in the volume fraction of stage 6 oocytes (Figure 6.2; Table 6.1; 6.2). By 10 days post-spawning, only the levels of  $E_2$  had decreased; whereas the levels of the T and  $Ca^{2+}$ , had still continued the increase to their peaks at day 10 after spawning (Figure 6.1; Table 6.1, 6.2).

#### 6.4.1.4 Spawning cycles and quality and quantity of eggs

The median of the first and the second spawning cycles was 13 days (range of 11 - 29 days;  $n = 20$  and 12 - 14 days;  $n = 6$  of the first and the second spawning cycles, respectively).

The quality and quantity of eggs at each spawning depended on the spawning history of the females. Average numbers of eggs were 418, 674 and 775 /female/spawn in the first, second and third spawning times, respectively. The egg numbers of the second and the third spawning females were significantly ( $P < 0.05$ ) higher than of the first spawning; but the differences between the second and the third spawning were not statistically ( $P > 0.05$ ) different.



In addition, the average fertilisation rates of the naturally spawned eggs were 55.0, 53.2 and 78.9% in the first, second and third spawning times, respectively, and these differences were not statistically ( $P>0.05$ ) different. Therefore, the spawning history had no bearing on these fertilisation rates (Figure 6.5a).

#### 6.4.2 Effect of 'Bi-weekly Bleeding Stress' on Spawning Cycles and Hormonal Profiles of the Individual *O. niloticus* (Experiment 2)

##### 6.4.2.1 Spawning cycles of non-mouthbrooding females

The spawning cycles of the eight individual females used in the present study, could be grouped into either short (9-16 days) or long (25 - 45 days) spawning cycles. The medians of the first, second and third spawning cycles were 13 (12 - 45), 28 (9 - 44) and 15 days (10 - 25), respectively (Figure 6.4). The overall median of all spawning cycles was 15 days (9 - 45). The spawning pattern of the individual females included both the short and long spawning cycles. For example, female no's 971 and 972 (Figure 6.3 b,c; Table 6.3) spawned at 12, 32, 13 days and 45, 15, 25 days, respectively. In comparison, female no. 834 showed only the short spawning cycles of 9 to 15 days (Figure 6.3a; Table 6.3).



#### 6.4.2.2 Spawning and the hormonal profiles

The relationship between spawning and peak levels of  $\text{Ca}^{2+}$ , T and  $\text{E}_2$  varied between individual females (Table 6.3; Appendix 12-19). The spawning of female no. 834 occurred after all the hormonal peaks. In some females (no's 971 and 972) the increase of hormones did not result in successful spawning. In these cases, spawning only occurred after several minor hormonal peaks had lapsed.

The levels of T and  $\text{E}_2$  during the three spawning cycles of the eight individual females were pooled according to the first spawning time occurred (first spawning). In the first spawning cycle, the average peak levels of T and  $\text{E}_2$  were 60.4 and 38.4 ng/ml, respectively; and 87% (7 out of 8) of the females spawned in the short spawning cycles (12 - 14 days) while the other 13% (1 out of 8) spawned in the long spawning cycle (45 days). In the second spawning cycle in comparison, the mean levels of T and  $\text{E}_2$  decreased to 34 and 28.6 ng/ml, and only 37 and 62.5% females spawned in the short (9 - 16 days) and long (27 - 44 days) spawning cycles, respectively. In the third cycle, the average peak levels of T and  $\text{E}_2$  reached to 45.3 and 24.4 ng/ml and 80 and 20% of the females spawned in the short (10 - 16 days) and long (25 days) spawning cycles, respectively. These peak levels of T showed no correlation to the  $\text{E}_2$  peaks and to the period of spawning cycles ( $r^2 = 0$ ;  $P > 0.05$ ).



#### 6.4.2.3 Quantity and quality of eggs

When the data from individual females were pooled, the duration of spawning cycles and hormonal levels had no significant bearing on the quantity of their eggs and fertilisation rates (Figure 6.5). The mean egg numbers per spawn of each females were 593, 772, 803 and 792 in the first, second, third and fourth spawnings, respectively. The fertilisation rates of the females at different spawning times were 47.4, 55.3, 53.4 and 84.8%, respectively (Figure 6.5).

A more detailed evaluation of data from individual females, however, suggested that egg quality was correlated with the peak levels of  $E_2$  (Figure 6.6b). The highest levels of this hormone resulted in high fertilisation rates and low fertilisation rates were affected by low  $E_2$  levels. The fertilisation rates of the females in each spawning cycle could be predicted from this following equation:

$$\text{Fert} = 127.3 \log E_2 - 135.1 \quad r^2 = 0.49; P = 0.00; df = 21$$
  
when:

Fert = fertilisation of eggs in a cycle (%)

$E_2$  = peak level of oestradiol-17 $\beta$  during  
the spawning cycle (ng/ml)



For example, in fish no. 834 (Figure 6.3a; Table 6.3), the highest  $E_2$  (48.3ng/ml) which occurred at her third spawning, resulted in the highest fertilisation rate (96%); and the medium (25 - 33ng/ml) and low (17.6ng/ml) peak of  $E_2$ , also corresponded with her medium (61 - 74%) and low (0%) fertilisation rates.

In comparison, the  $E_2$  levels in the long spawning cycles (32 - 45 days of female no's 971 and 972, respectively), ranged between 24.3 - 32.1ng/ml and 21.6 - 32.6ng/ml and their fertilisation rates were 68 and 47.9%, respectively. In female no's 970 and 972, the fertilisation rates increased from 44 - 61.7% to 78 - 95%, when the  $E_2$  levels ranged between 38 - 55.2 ng/ml, during the long breeding cycles of 25 - 37 days (Figure 6.3c; Table 6.3 and Appendix 14).

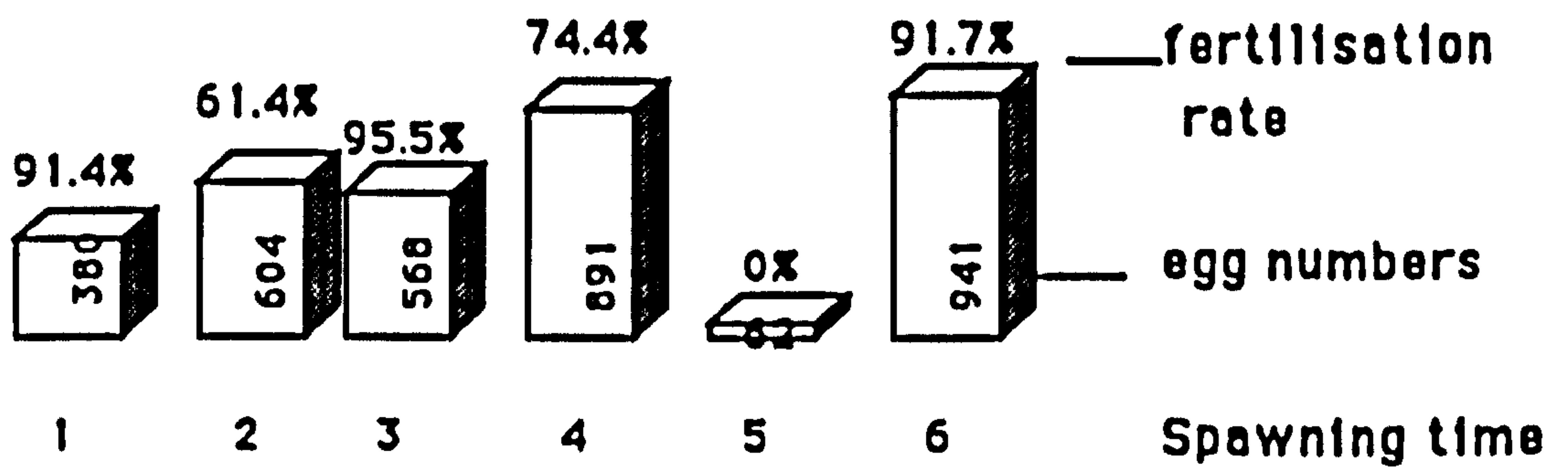


Table 6.3: Peak levels of testosterone (T), oestradiol-17 $\beta$  (E<sub>2</sub>) and natural fertilisation rates of non-mouthbrooding *O. niloticus* females during each spawning cycles. Data were recorded from eight individual females.

| Fish no | Cycles | Peak of steroid levels (ng/ml) |                       | Fertilisation rate (%) | Spawning cycle (days) |
|---------|--------|--------------------------------|-----------------------|------------------------|-----------------------|
|         |        | Testosterone                   | Oestradiol-17 $\beta$ |                        |                       |
| 818     | 1      | 48.5                           | 39.1                  | 2.2                    | 14                    |
|         | 2      | 48.0                           | 27.0                  | 0.0                    | 16                    |
| 833     | 1      | 76.4                           | 29.8                  | 0.9                    | 13                    |
|         | 2      | 63.0                           | 39.9                  | 53.4                   | 45                    |
|         | 3      | 37.8                           | 45.7                  | 95.5                   | 15                    |
| 834     | 1      | 88.2                           | 32.6                  | 61.4                   | 12                    |
|         | 2      | 34.9                           | 48.3                  | 95.5                   | 9                     |
|         | 3      | 57.1                           | 25.2                  | 74.4                   | 10                    |
|         | 4      | 33.6                           | 17.6                  | 0.0                    | 12                    |
|         | 5      | 121.8                          | 21.3                  | 91.7                   | 15                    |
| 970     | 1      | 84.6                           | 52.5                  | 61.7                   | 12                    |
|         | 2      | 58.0                           | 55.2                  | 95.5                   | 37                    |
|         | 3      | 54.9                           | 39.7                  | 90.4                   | 16                    |
| 971     | 1      | 52.5                           | 28.8                  | 84.3                   | 12                    |
|         | 2      | 63.0                           | 32.1                  | 68.1                   | 32                    |
|         | 3      | 65.1                           | 48.3                  | 85.5                   | 13                    |
| 972     | 1      | 62.6                           | 32.6                  | 47.9                   | 45                    |
|         | 2      | 30.2                           | 37.0                  | 44.2                   | 15                    |
|         | 3      | 58.3                           | 38.0                  | 78.0                   | 25                    |
| 974     | 1      | 52.3                           | 45.7                  | 92.3                   | 13                    |
|         | 2      | 62.8                           | 33.7                  | 65.0                   | 27                    |
| 986     | 1      | 36.0                           | 46.8                  | 92.0                   | 13                    |
|         | 2      | 42.1                           | 32.6                  | 54.0                   | 29                    |



(a)



(b)

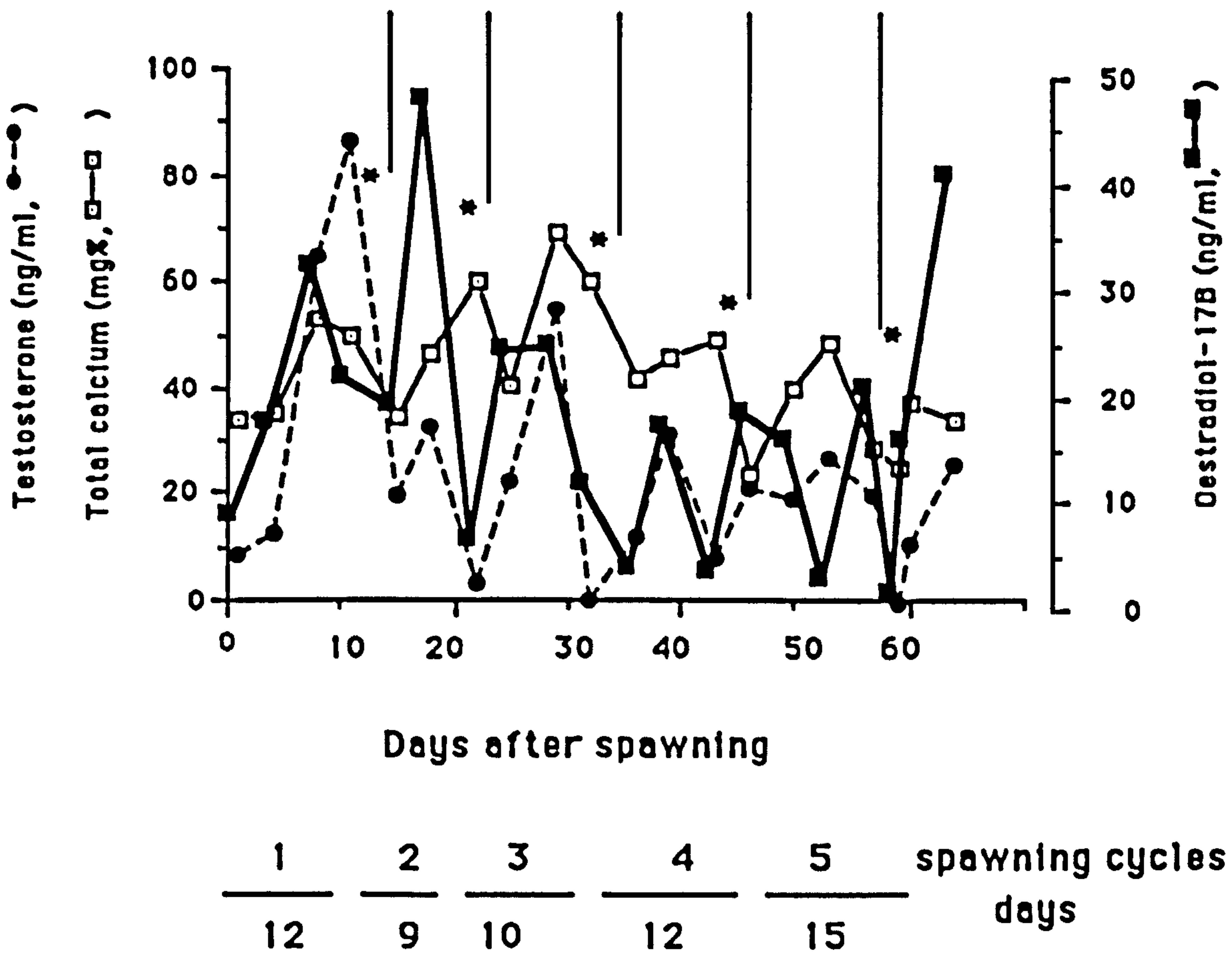


Figure 6.3a: Changes in serum total calcium ( $\text{Ca}^{2+}$ ), oestradiol-17 $\beta$  ( $\text{E}_2$ ), testosterone (T) and egg quality during five spawning cycles of non-mouthbrooding *O. niloticus* female no. 834 : (a) Egg numbers and fertilisation rates and (b) Profiles of total calcium ( $\text{Ca}^{2+}$ ), testosterone (T) and oestradiol-17 $\beta$  ( $\text{E}_2$ ). (\*) Indicates spawning time.



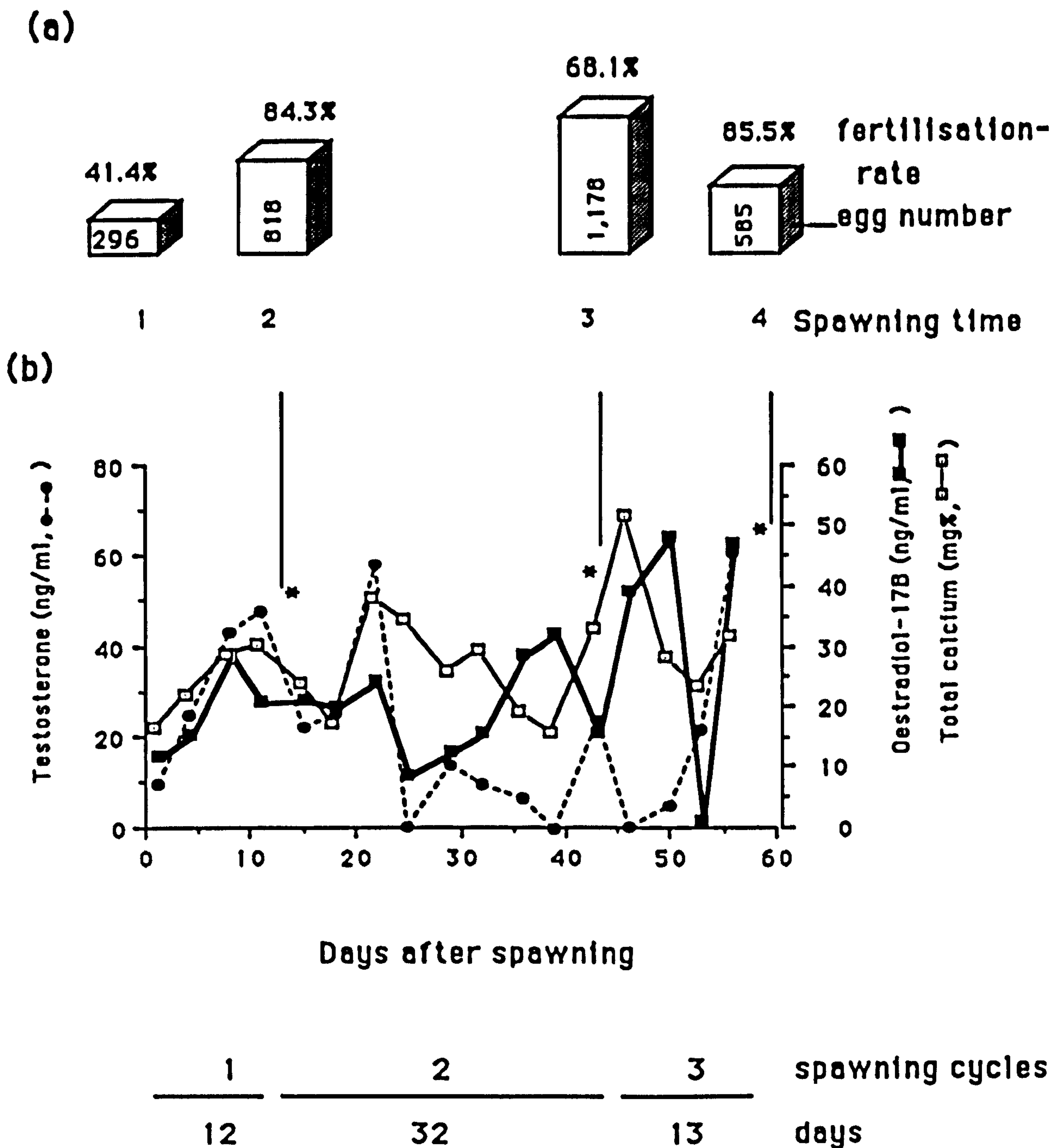


Figure 6.3b: Changes in serum total calcium ( $\text{Ca}^{2+}$ ), oestradiol-17 $\beta$ , testosterone (T) and egg quality during three spawning cycles of non-mouthbrooding *O. niloticus* no. 971 : (a) Egg numbers and fertilisation rates and (b) Profiles of total calcium ( $\text{Ca}^{2+}$ ), testosterone (T) and oestradiol-17 $\beta$  ( $\text{E}_2$ ). (\*) Indicates spawning time.



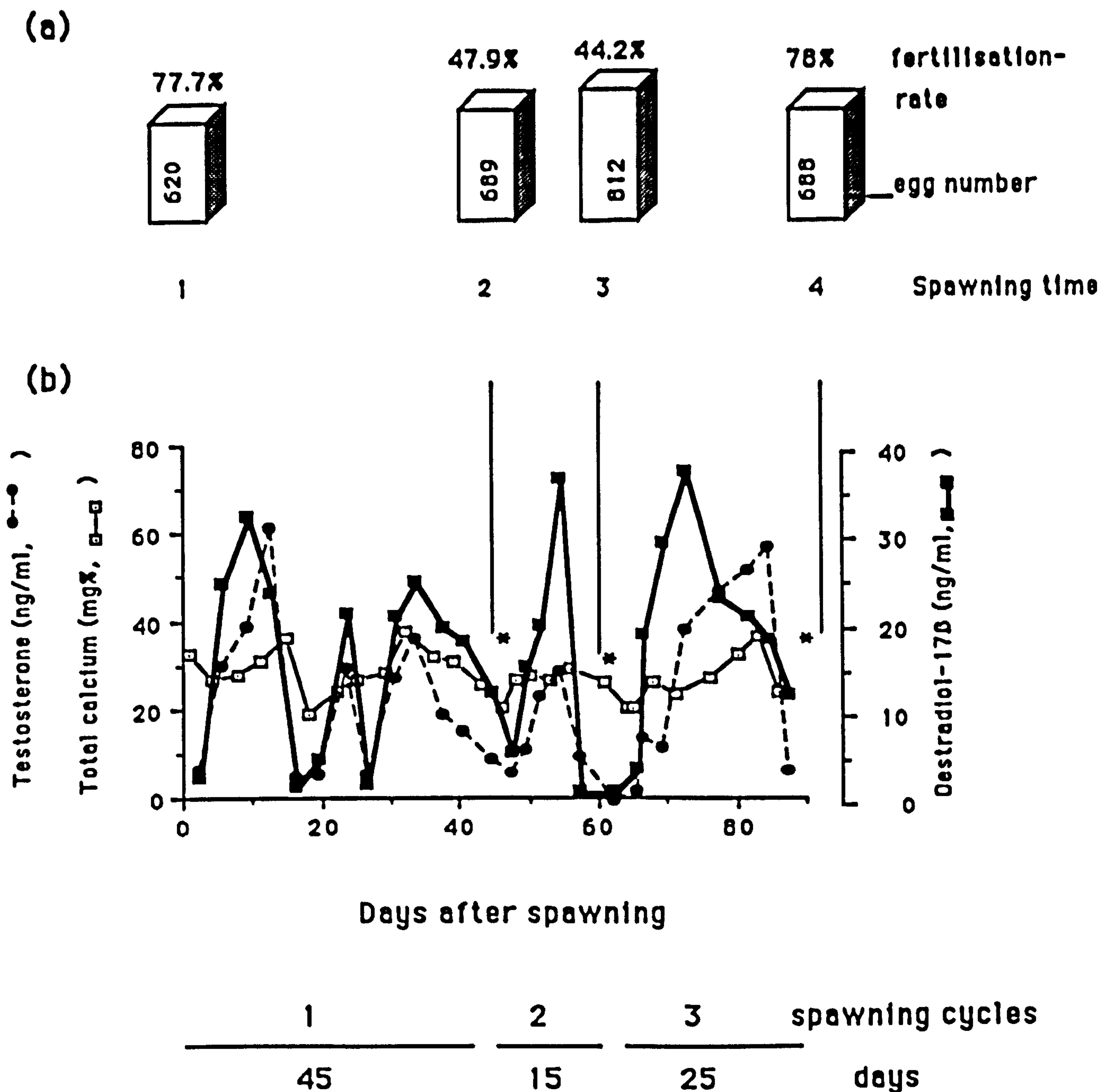


Figure 6.3c: Changes of serum total calcium ( $\text{Ca}^{2+}$ ), oestradiol-17 $\beta$  ( $\text{E}_2$ ), testosterone (T) and egg quality during three spawning cycles of non-mouthbrooding *O. niloticus* no. 972 : (a) Egg numbers and fertilisation rates, (b) Profiles of total calcium ( $\text{Ca}^{2+}$ ), testosterone (T) and oestradiol-17 $\beta$  ( $\text{E}_2$ ). (\*) Indicates spawning time.



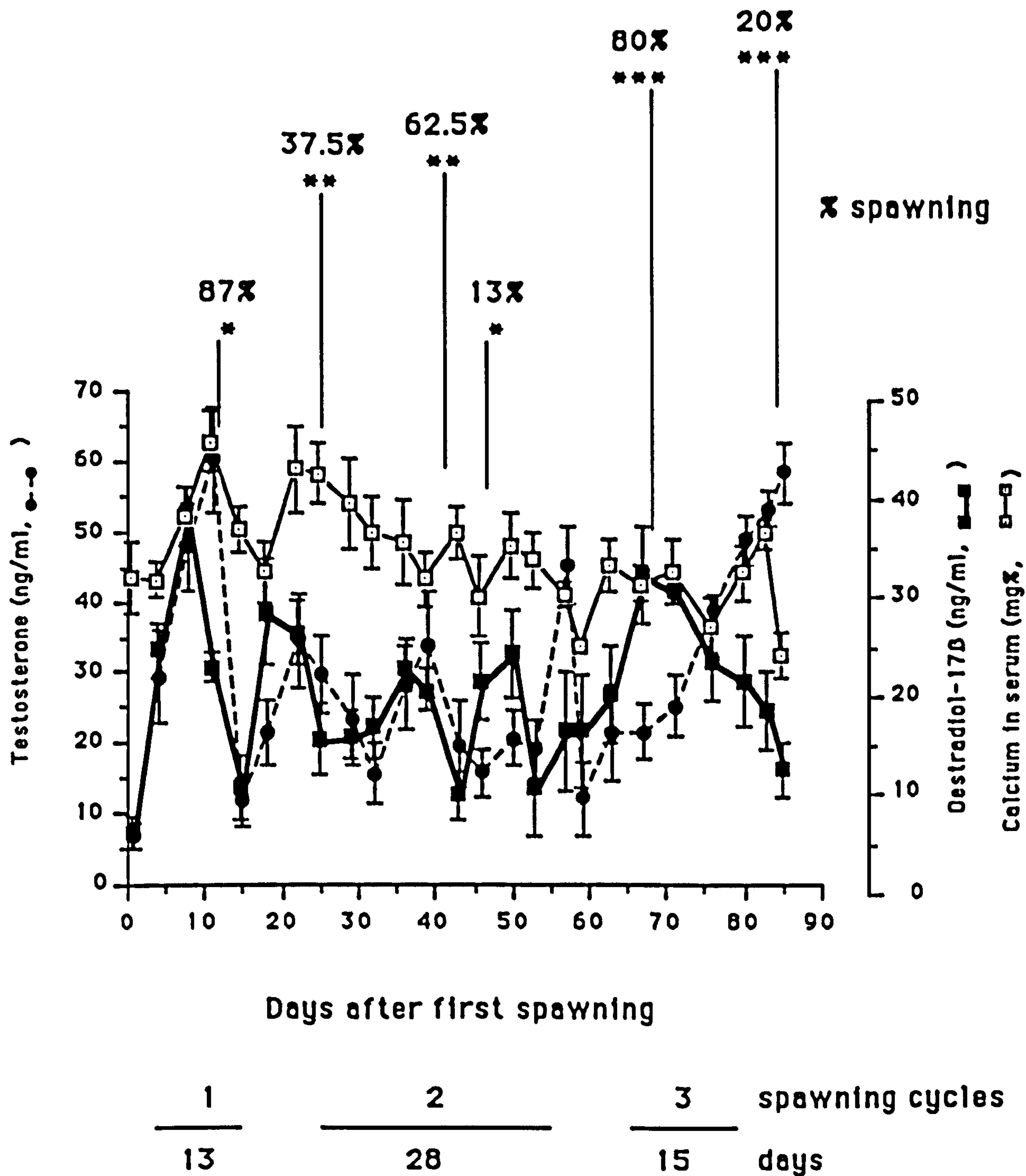


Figure 6.4: Changes in average ( $\pm$ SE) levels of total calcium ( $\text{Ca}^{2+}$ ), testosterone (T) and oestradiol-17 $\beta$  ( $\text{E}_2$ ) (pooled data from 8 individual females) coincide with percentage of successful spawning at each median time of the spawning cycle of non-mouthbrooding *O. niloticus*. (\*) Indicates first spawning; (\*\*) indicates second spawning; (\*\*\*) indicates third spawning.



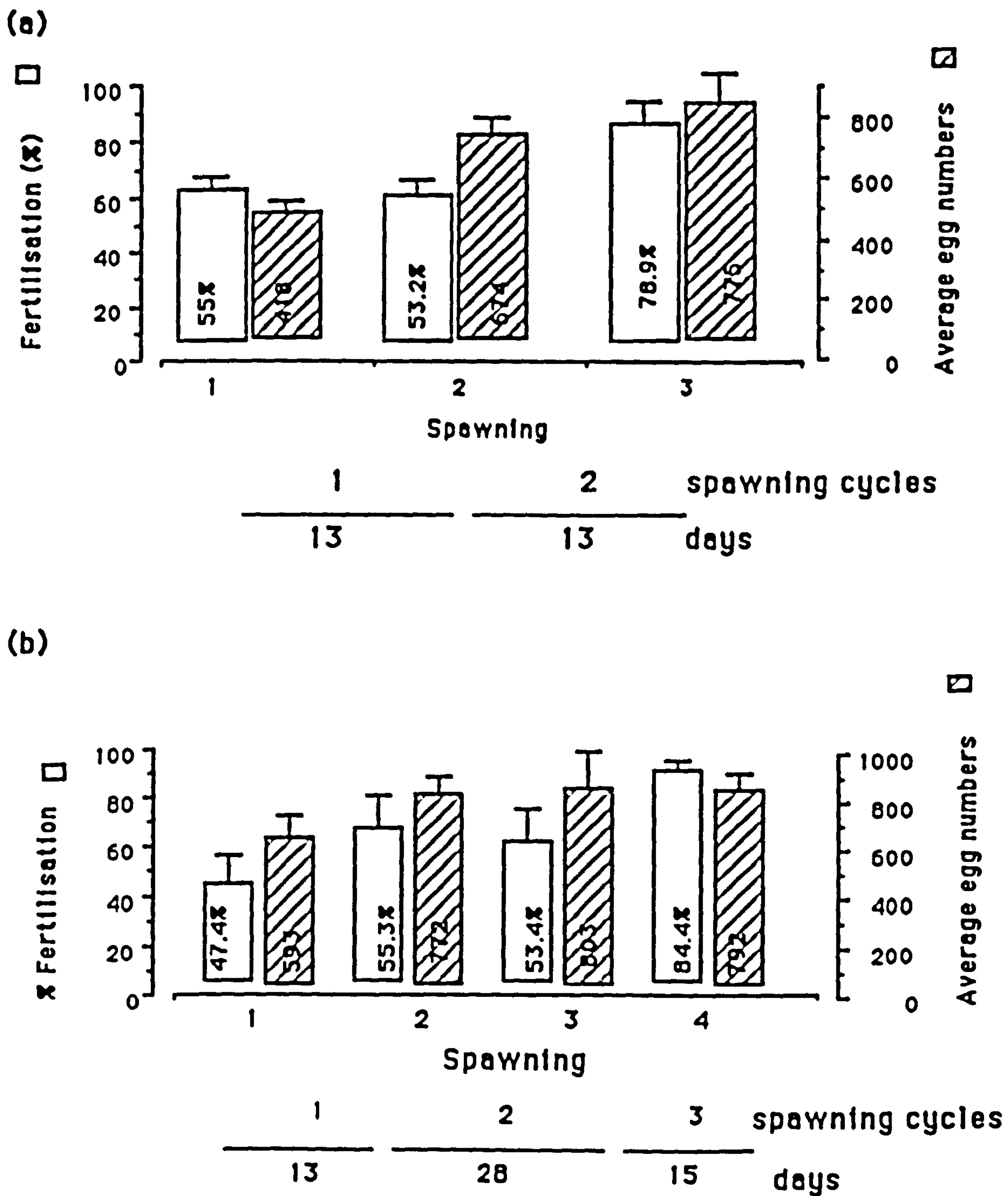


Figure 6.5: Comparison of average egg numbers, fertilisation and median times of spawning cycles of non-mouthbrooding *O. niloticus* females. (a) data from section 6.4.1 where the fish were bled only once in their life when sacrificed, (b) data pooled from the eight individual females (section 6.4.2) which were bled bi-weekly over a period of 43 to 85 days.



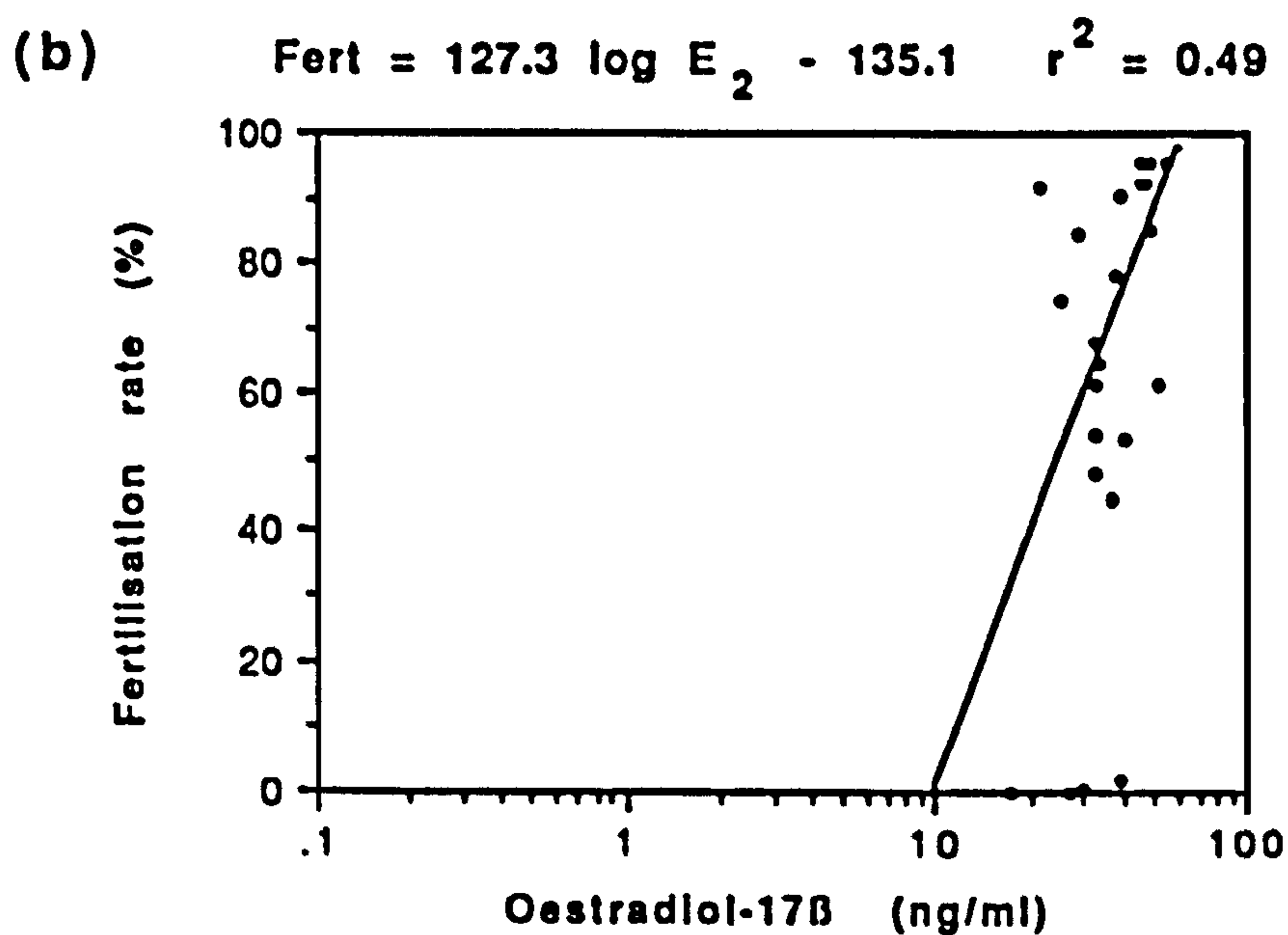
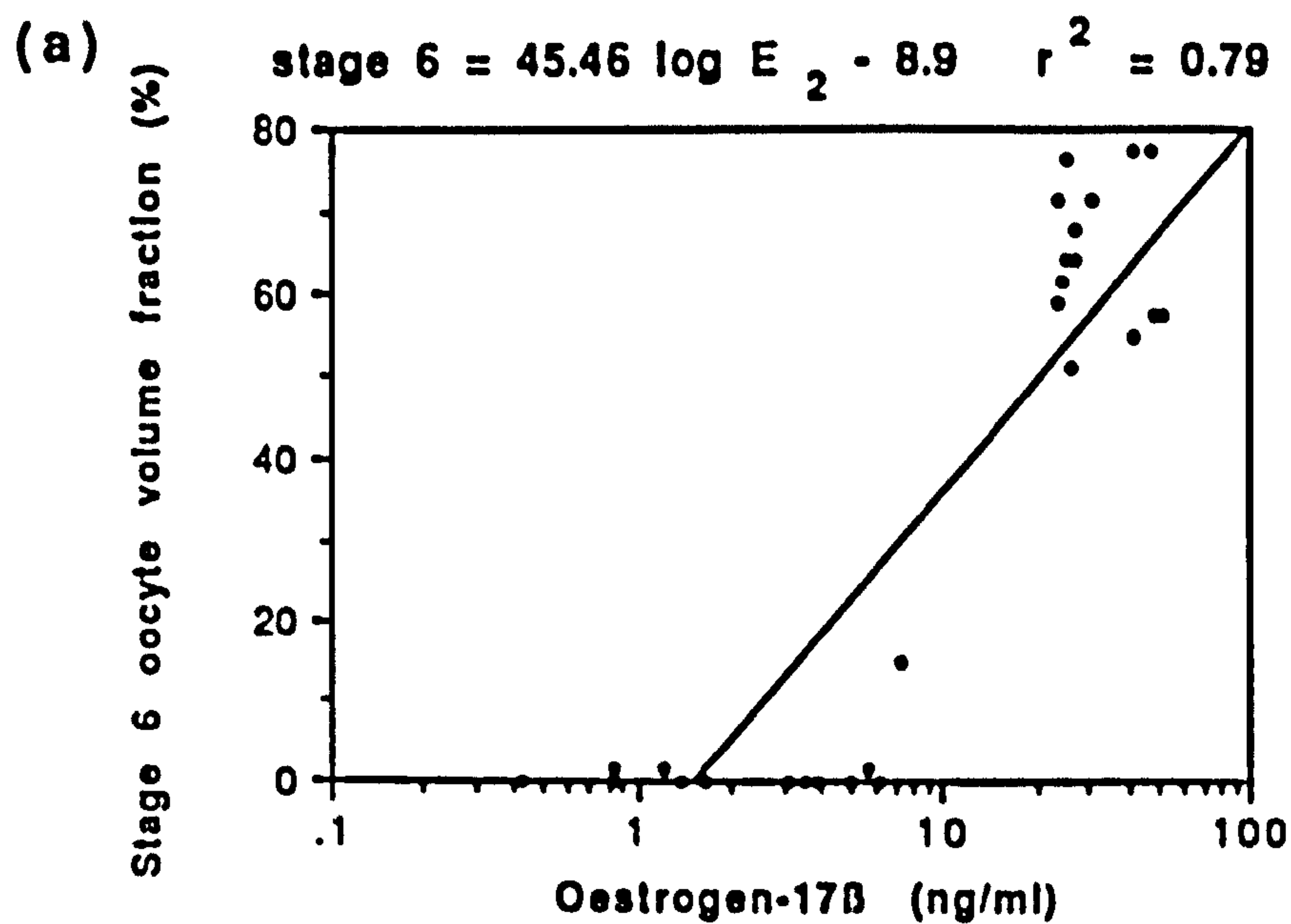


Figure 6.6: (a) Relationship between oestradiol-17 $\beta$  and stage 6 oocyte volume fraction (df = 23); (b) Relationship between oestradiol-17 $\beta$  and fertilisation rate (df = 21); of the eight non-mouthbrooding *O. niloticus* females. The fish were stocked at the density of 3 females : 1 male and allowed to spawn naturally in 2 m diameter fibre glass tanks.



Table 6.4: Egg numbers, fertilisation rates and spawning cycles of the eight individual *O. niloticus* females

| Spawning history |       |          |                  |        |          |                  |       |          |                  |        |          |
|------------------|-------|----------|------------------|--------|----------|------------------|-------|----------|------------------|--------|----------|
| Fish No.         | First |          |                  | Second |          |                  | Third |          |                  | Fourth |          |
|                  | Eggs  | Fert (%) | Day <sup>1</sup> | Eggs   | Fert (%) | Day <sup>1</sup> | Eggs  | Fert (%) | Day <sup>1</sup> | Eggs   | Fert (%) |
| 834              | 380   | 91.4     | 12               | 604    | 61.4     | 9                | 568   | 59.5     | 10               | 891    | 74.4     |
| 833              | 1056  | 24.0     | 13               | 1207   | 0.9      | 44               | 1245  | 53.4     | 15               | 939    | 95.5     |
| 970              | 347   | 77.2     | 12               | 736    | 61.7     | 37               | 876   | 95.5     | 16               | 856    | 90.4     |
| 971              | 296   | 41.4     | 12               | 818    | 84.3     | 32               | 1178  | 68.1     | 13               | 585    | 85.5     |
| 972              | 620   | 77.7     | 45               | 689    | 47.9     | 15               | 812   | 44.2     | 25               | 688    | 78.0     |
| 818              | 814   | 30.3     | 14               | 622    | 2.2      | 16               | 64    | 0        | -                | -      | -        |
| 974              | 491   | 36.7     | 13               | 563    | 92.3     | 27               | *     | *        | -                | -      | -        |
| 986              | 743   | 0.88     | 13               | 934    | 91.9     | 29               | 876   | *        | -                | -      | -        |
| mean             | 593.4 | 47.4     | 13 <sup>2</sup>  | 771.6  | 55.3     | 28 <sup>2</sup>  | 803   | 53.4     | 15 <sup>2</sup>  | 791.8  | 84.8     |
| ±S.E             | ±93.6 | ±11.1    | 12-45            | ±75.6  | ±13      | 9-44             | ±150  | ±12.9    | 10-25            | ±66.8  | ±3.9     |

\*\*\* Indicates missing data.

\*-\* Indicates no data.

<sup>1</sup> Indicates period in each spawning cycle. <sup>2</sup> Indicates median and minimum-maximum.



## 6.5 DISCUSSION

In the present study an increase in mature stage 6 oocytes corresponded with the increase of gonadosomatic index (GSI). The GSIs of *O. niloticus* females during each spawning cycle increased significantly at day 5 (2.5 - 3%) and peaked (3.9 - 4.5%) by day 10 after spawning. These GSI levels were similar to the maximum GSIs of the same species from Lake Nasser during the natural breeding season (3.3%; Latif and Rashid, 1972). This overall trend in the rise of the GSI prior to spawning was also similar to the seasonally breeding teleosts, such as Indian catfish (Ghosh and Kar, 1952), grey mullet (Abraham and Blanc, 1966), soleidae (Deniel, Blanc and Rodriguez, 1989), freshwater catfish, *Tandanus tandanus* (Davis, 1977) and goldfish (Kobayashi, Aida and Hanyu, 1986). The *O. niloticus* ovaries had, therefore, completed vitellogenesis and maturation by day 10 after spawning.

Stereological and histological techniques were used in the present study to quantify ovarian recrudescence as the ovarian volume fraction of the different oocyte stages. The morphological changes in *O. niloticus* ovary were similar to those of other poikilothermic species but the duration of the spawning cycle varied. In nature, the volume fraction of mature gametes of mollusc breeders peaked once a year during the breeding season (Lowe et al., 1982; MacDonald and Thompson, 1986). In *O. niloticus* of the present study,



oocyte recrudescence was completed in a relatively short period of 10 days. In day 1 post-spawned ovaries, post-ovulatory follicles, atretic and stages 5 and 6 oocytes occupied a total of 81.8% of the ovary. Within days 5 and 10 after spawning, only stage 6 oocytes increased significantly ( $P < 0.05$ ) and occupied approximately 58 - 60% and 65 - 72% of the ovary, respectively.

Using similar techniques it was demonstrated that in annual spawners like mussel and giant scallop, the volume fractions of mature (ripe) gonads took a year to develop and occupy from 70 - 80% and 75 - 95% of the ovaries, respectively (Lowe et al., 1982; MacDonald and Thompson, 1986). In contrast, the multiple spawners in the present study (the non-mouthbrooding *O. niloticus* females) required approximately 10 days after spawning to develop a new batch of mature stage 6 oocytes and their volume fractions occupied 64.7 - 72% of the ovaries. These females were then able to spawn at a median time of 13 days.

As maturation proceeds, significant changes occur in the reproductive system as well as the metabolism of the fish. The changes in serum  $\text{Ca}^{2+}$ , T and  $\text{E}_2$  are well reported parameters associated with oocyte maturity and GtH levels (Whitehead et al., 1978a,b; 1983; Scott et al., 1983; Crim and Idler, 1978; Bromage and Cumaranatunga, 1988). In the present study, the significantly higher  $\text{Ca}^{2+}$ , T and  $\text{E}_2$  levels at day 5 after spawning confirmed that the oocytes were in



vitellogenesis. At day 10 after spawning, the levels of T and  $\text{Ca}^{2+}$  increased whereas  $\text{E}_2$  decreased. The differences between the steroid hormones at days 5 and 10 after spawning, however, were not significant. A similar increase in these steroid profiles during maturation was reported for winter flounder, *P. americanus* (Campbell et al., 1976), grey mullet, *M. cephalus* (Azoury and Eckstein, 1980) and goldfish (Kobayashi, Aida and Hanyu, 1986).

Maturity of non-mouthbrooding *O. niloticus* females may be quantified by the presence of stage 6 oocytes in the ovary. Stage 6 oocyte volume fractions were positively correlated with serum  $\text{E}_2$  levels during the spawning cycle (Figure 6.6a). Therefore, the investigation of serum  $\text{E}_2$  levels could be used to determine the % of the ovary occupied by stage 6 oocytes without sacrifice females (Figure 6.6a). The  $\text{E}_2$  levels in the present study were similar to the  $\text{E}_2$  levels during puberty in the females (*O. niloticus*) described in chapter 5. These  $\text{E}_2$  profiles among tilapia species, however, were different to those reported for non-mouthbrooding *O. mossambicus* by Smith and Haley (1988). In their studies they observed twin peaks of  $\text{E}_2$  first at day 10 and then at day 25 after spawning. In addition, when the *O. niloticus* females were sampled according to their spawning behaviour, they showed the  $\text{E}_2$  peak during the pair forming behaviour (Rothbard, Ofir, Levavi-Sivan and Yaron, 1991). In contrast, the  $\text{E}_2$  profiles in the present study, were similar to the profiles for salmonids and goldfish (Scott et al.,



1983; Kobayashi et al., 1986; 1988; 1989). These studies showed an increase of  $E_2$  during vitellogenesis, followed by a decrease of  $E_2$  before spawning. For example in rainbow trout, *Oncorhynchus mykiss*, the decrease of  $E_2$  occurred approximately 16 days prior to ovulation (Scott, et al., 1983). In summary, the results in the present study confirmed that the ovarian recrudescence and hormonal peaks in *O. niloticus* were attained within 10 days after spawning but subsequent spawning occurred at the overall median time of 13 days (11 - 29 days).

The spawning cycles varied between individual females. The effect of bi-weekly bleeding trial (experiment 2), suggested that the stress associated with the bleeding had no noticeable effects on their spawning cycles. In these fish, spawning occurred between 13 to 28 days (short and long spawning cycles, respectively) and were similar to the range reported by Fishelson (1966), Mires (1982) and section 6.4.1 of the present study.

These spawning cycles (short or long) of non-mouthbrooding *O. niloticus* in the present study may be due to the presence of a semilunar or lunar cycle. Although the females in the present study were kept under the controlled conditions, of 12L:12D, their spawning pattern corresponded to semilunar or lunar cycle. Lunar influences on reproduction had been reported for the wild tilapias, in which the high proportion of mature female gonads, pair forming and



parental care were found during full moon rather than during new moon (Schwanck; 1987 and Nakai, Yanagisawa, Sato, Niimura and Gashagaza, 1990).

The timing of the concomitant rise and the peak levels of T and E<sub>2</sub>, however, varied between individual females (Figure 6.3 a-c; Table 6.3) and therefore, the spawning cycle between individual females varied. These spawning cycles could be grouped as either short (9 - 16 days) or long (25 - 45 days) depending on the peak levels of T and E<sub>2</sub>. At the high levels (T = 60.4 and E<sub>2</sub> = 38.4ng/ml), 87% of the females spawned in short spawning cycles (12 - 14 days).

In contrast, the females which had lower peak levels of T and E<sub>2</sub> tended to spawn in long spawning cycles. For example, when average peak levels of T and E<sub>2</sub> were 34.6 and 28.6ng/ml, respectively, 37.5 and 62.5% of the females spawned in short and long spawning cycles, respectively. These differences in the spawning cycles were observed despite the fact that the stereological study on ovarian recrudescence (section 6.4.1) showed that within 10 days after spawning, ovaries of these females contained 65 - 72% stage 6 oocytes, and were thus, mature females. It is possible that spawning in these fish was not triggered due to the suboptimal levels of T and E<sub>2</sub>.

The results in the present study indicates that the fish



may not necessary spawn at every peak of their  $\text{Ca}^{2+}$ , T and  $\text{E}_2$  profiles, and that successful spawning may be related to the peak levels of the  $\text{E}_2$  and T. The result in the present study corresponds to Santos, Furukawa, Kobayashi, Bando, Aida and Hanyu (1986) who reported a small and rhythmical increase of T and GtH in unovulated female carp, *Cyprinus carpio*. Similar results were also found in coho salmon; fish with low T levels, took a longer period of about 41 to 49 days for ovulation when compared to the shorter spawning period of 10 to 14 days for fish with higher T levels (Fitzpatrick, Redding, Ratti, and Schreck, 1987).

To obtain a clearer understanding of the timing of female spawning, additional information on hormones like GtH and  $17\alpha,20\beta\text{-P}$ , which rise sharply prior to spawning in many teleost species, such as rainbow trout, common carp, yellow perch, amago salmon and ayu (Scott et al., 1983; Santos et al., 1986; Kobayashi et al., 1986; Goetz and Theofan, 1979; Nagahama et al., 1980; Suzuki et al., 1981), may be required. Unfortunately as these assays were not available at the Institute of Aquaculture, it was not possible to measure these hormones in the present study. The *O. niloticus* females in this study, may have lacked appropriate GtH and/or  $17\alpha,20\beta\text{-P}$  levels during the long spawning period of 28 days (9 - 44 days) and this may have also prevented the females from spawning after ovarian maturation was completed. In these females atresia subsequently occurred. The processes of oocyte resorption and recruitment may take



another 10 days (section 6.4.1); the ovaries may then complete maturity and the fish may spawn successfully in the second cycle.

The number of eggs per spawn from each female may depend on their spawning history. The average number of eggs at the second (674) and third (775) spawning were significantly higher than those from the first (418) spawning (Figure 6.5a). Similar evidence was also reported for other tilapia species (Peter, 1983).

The differences in egg numbers at each spawning time may coincide with the ovarian structures after spawning. After the first spawning, ovaries contained the highest volume fraction of atretic oocytes (21.4%) compared to 17.9 and 16.6% in the second and third spawning, respectively. The volume fraction of post-ovulatory follicles, on the other hand, increased from 16.2% of the first spawning to 18.8 and 27.4% in the second and third spawning, respectively. This denotes that the females who have spawned (second or third spawning time) may successfully ovulate more eggs than those that have never spawned (the first spawning).

The peak levels of  $E_2$  was positively correlated with the volume fraction of stage 6 oocytes and egg fertilisation rates. Therefore, the measurement of serum  $E_2$  may indicate the ovarian maturity and quality of subsequently spawning eggs. For example, in this study when peak levels of  $E_2$



prior to spawning (Figure 6.6b) were high ( $E_2$  = 45.7 - 55.2 ng/ml), the fertilisation rates ranged between 91.7 - 95.5%, whereas lower levels of  $E_2$  (17.6 - 33ng/ml) resulted in the fluctuated fertilisation rate between 0 - 74% (Figure 6.3a-c).



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## CHAPTER 7

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## 7. EFFECT OF D-ALA<sup>6</sup>, DES GLY<sup>10</sup>-LUTEINIZING HORMONE-RELEASING HORMONE ETHYLAMIDE (LHRH) ADMINISTRATION AND SOCIAL INTERACTION ON THE INTERSPAWNING PERIOD OF *O. niloticus*

### 7.1 INTRODUCTION

The efficacy of hormonal techniques for inducing and synchronizing of fish ovulation and spawning in commercial hatcheries has dramatically improved over the last decade. Much of this success has been derived from a better understanding of the hormonal control of oogenesis.

The ovarian development of teleosts is under the control of several different hormones. These hormone are produced at different sites in the fish and each hormone plays a critical role in co-ordinating the development of oocytes through the series of interrelated stages (Figure 7.1).

Various crude and purified hormones which induce fish to spawn are well documented, for example, pituitary hypophysation (pituitary extract), mammalian gonadotropins (HCG, PMS), gonadotropin releasing hormone (GnRH), luteinizing hormone-releasing hormone (LHRH) and their analogues (Fontaine, 1976; Chaudhuri, 1976; Yamazaki, 1976; Lam, 1982; Donaldson and Hunter, 1983; Lam, 1985; Crim, 1985; Donaldson, 1986). All these have been reported to have some measure of success in induced spawning in teleosts.



Moreover, a variety of methodologies and hormones are used to control the spawning of fish. Most of these hormones or neurotransmitters stimulate the release of gonadotropins (GtHs) or inhibit the gonadotropin-releasing inhibitor (GRIF), which causes the pituitary to release more GtH into the blood stream. When the GtHs reach the gonads e.g., ovary, they eventually result in the migration of the germinal vesicle of mature oocytes and stimulates the thecal and the granulosa cells to produce maturation inducing steroid (MIS) (Nagahama, 1983) identified as  $17\alpha$ ,  $20\beta$ -dihydroxy-4-pregnen-3-one ( $17\alpha, 20\beta$ -P) (Scott and Canario, 1987). The MIS ( $17\alpha, 20\beta$ -P) subsequently induces germinal vesicle break down (GVBD), hydration, coalescence of yolk granules or yolk globules, ovulation and oviposition.



## INDUCED MATURATION AND OVULATION

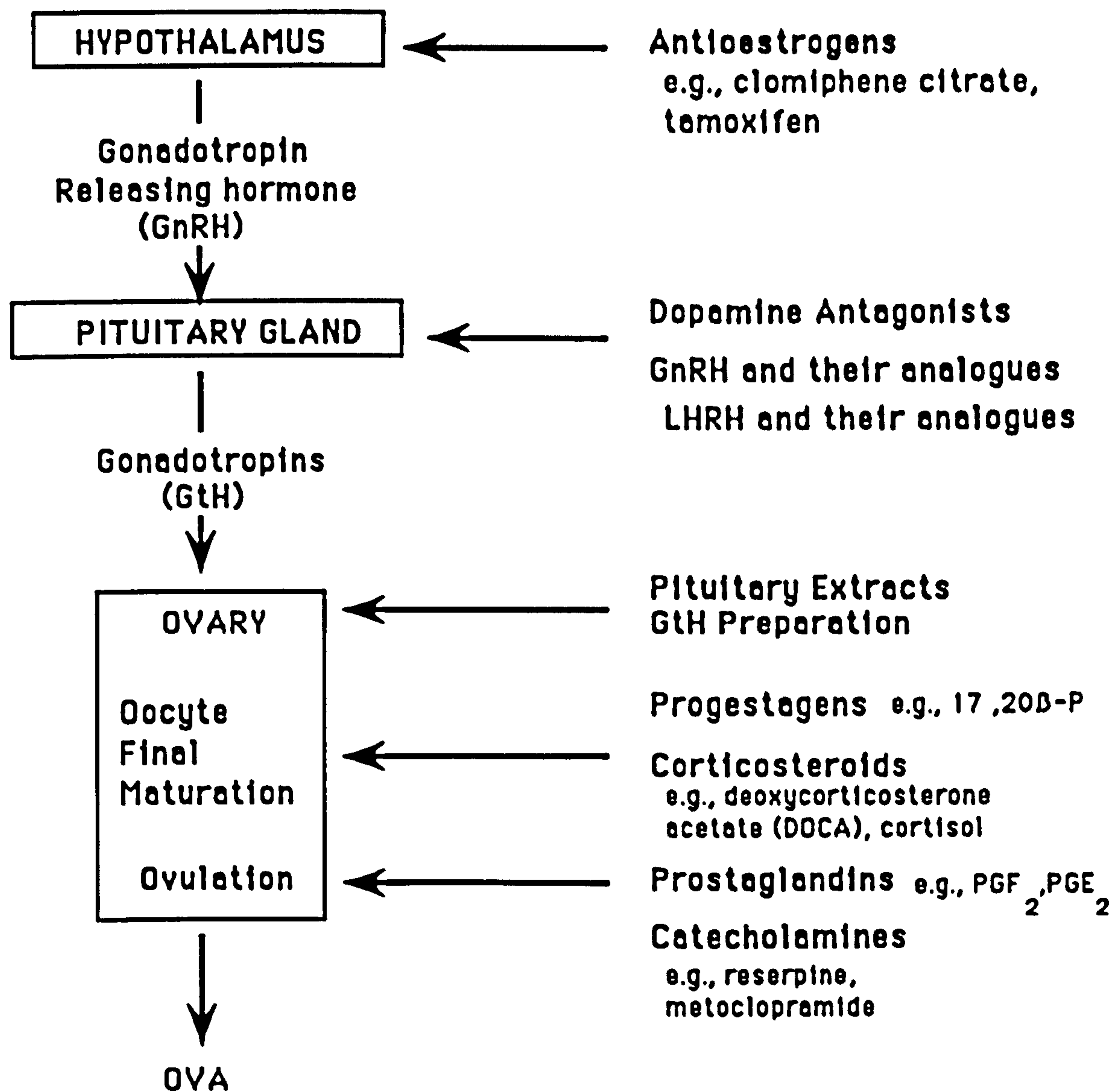


Figure 7.1: Sites of action of specific endocrine methodologies for the control of maturation and ovulation in salmonids. Modified from Donaldson (1986).



## **7.1.2 Hormonal Technologies Used to Induce Teleosts to Spawn**

### **7.1.2.1 Fish pituitary extracts (Hypophysation)**

The use of pituitary extracts to induce spawning of fish was initiated by Houssay in 1931 and has since been extensively reviewed and widely used to induce spawn in many fish species e.g., Indian carps, Chinese carps and catfish, etc (Fontaine, 1976; Chaudhuri, 1976; Yamazaki, 1976; Lam, 1982; Donaldson and Hunter, 1983; Lam, 1985).

The pituitary glands are generally obtained from sexually maturing or mature donor fish which may be of the same (homologous) or different species (heterologous) to the recipient. Fresh pituitary glands or glands preserved in acetone or alcohol have been used to induce fish to spawn successfully in particular to cyprinids (Donaldson and Hunter, 1983; Yaron and Zohar, 1993). This pituitary hypophysation generally results in a surge of GtH in the recipient females and this stimulates oocyte maturation and ovulation. Efficacy and dosage of this hypophysation in teleosts can be variable and influence the quality of eggs. Relatively high dosages of the pituitary are required e.g., 3-5 mg/kg body weight of carp (Lam, 1982; 1985; Donaldson and Hunter, 1983; Billard, 1989; Zohar, 1989) for using the pituitary from different species. This heterologous hypophysation may stimulate more immunity of the recipient



which may then need higher doses or become resistant to any subsequent hypophysation treatment (Lam, 1982). The heterologous hypophysation may only result in partial ovulation, translucent oocytes and incomplete embryonic development. This general failure has been attributed to inappropriate food, insufficient quality and quantity of endocrine system in the fish or hypophysation used (Billard, 1993).

Although pituitary extract is the simple and the most practical technique, it has several other drawbacks. For example, quality of gonadotropic content in the pituitary gland varies between seasons and species. Although this technique had been improved by using GtH-calibrated carp pituitary extract which contains purer GtH and known potency, this treatment is mostly effective when used in related species of cyprinids (Yaron and Zohar, 1993). When using this calibrated carp pituitary extract in the other teleost families, it is difficult to standardize the dosage of the hypophysation used. In addition, the closely-related pituitary hypophysation also difficult to obtain. The success of the pituitary hypophysation technique, generally depends on the maturity of recipient fish and the skill of the users (Lam, 1982; 1985).



#### 7.1.2.2 Human chorionic gonadotropin (HCG)

To overcome the various drawbacks of treatment with the pituitary gland, human chorionic gonadotropin (HCG) was selected and used to replace or to be used in combination with the pituitary gland (Lam, 1982). Although HCG is the heterologous placental gonadotropin, the use of the HCG alone or the combination of HCG and pituitary gland has been reported to induce ovulation and spawning in a number of fishes (Lam, 1982; Donaldson and Hunter, 1983; Lam, 1985). This technique, however, also affects the immune system of the fish. In addition, the dosage required may depend on species, gonadal and endogenous stages of the fish (Lam, 1982; 1985).

#### 7.1.2.3 Partially purified or purified fish gonadotropin preparations

Recently, a number of partially purified or purified fish gonadotropin preparations from salmon, carp, rainbow trout and puntius have become available in acetone-dried or lyophilized forms from several laboratories. Due to its availability, the salmon gonadotropin; SG-G100, has been most widely tested in inducing cultured fish species to spawn (Donaldson and Hunter, 1983).



Studies on the neuro-endocrine regulation of goldfish (Peter, 1983), have indicated that there are two hypothalamic hormones which control GtH release from the pituitary gland; gonadotropin releasing hormone (GnRH) and dopamine which antagonizes the function of GnRH. The GnRH stimulates the fish pituitary gland to release its GtH. This GtH has no species-specificity and has no effect on the immune system of the fish (Yaron and Zohar, 1993).

#### 7.1.2.4 Gonadotropin releasing hormones (GnRH), luteinizing hormone-releasing hormone (LHRH) and their analogues

The higher degree of species-specificity of fish gonadotropins (GtHs) and the expense involved in obtaining purer preparations of fish GtHs, led to the search for other alternative hormonal therapies to manipulate fish ovulation and spawning. Due to GnRH or LHRH being a small molecular structure composed of ten peptides, it is easily synthesized as a commercial product. In recent years some of the synthetic decapeptides such as LHRH, GnRH and their analogues have been found to be effective in inducing spawning teleost species such as, salmonids, cyprinids, mullet, milkfish, seabream, seabass, plaice, flounder, catfish, sablefish, Japanese eel, white sturgeon and herring (Zohar, 1989; Peter, Lin, van der Kraak and Little, 1993).



The effectiveness of GnRH-based spawning technology depends on: 1) interaction between GnRH and the gonadotropin releasing inhibitory factor (GRIF) in the regulation of GtHs release and ovulation, 2) structure-activity relationship of GnRH and its analogues and lastly 3) mode of GnRH administration to the fish (Zohar, 1989).

The analogues of GnRH and LHRH (GnRHa and LHRHa), act as super active ovulating agents in cultured fish species. The most well known LHRHa used include des-Gly<sup>10</sup> [D-Ala<sup>6</sup>]LH-RH-ethylamide, des-Gly<sup>10</sup> [D-Ser(But)<sup>6</sup>]LH-RH-ethylamide, [6-(D-2-naphthylalanine)]LH-RH, des-Gly<sup>10</sup> [D-Trp<sup>6</sup>]LH-RH-ethylamide, D-Ser<sup>6</sup> LH-RH-ethylamide and [D-Ala<sup>6</sup>,Pro<sup>9</sup>-N-ethylamide]LH-RH all of which are available commercially.

In conclusion, the advantages of LHRH, GnRH and their analogues include: 1) they stimulate GtH secretion directly, 2) they can be synthesized and obtained in a pure form, 3) they exhibit a low degree of biological species specificity, 4) the mammalian GnRH is effective in a variety of fish species, thus they are commercially available, 5) they are very small peptides, thus they are non-immunostimulative agent or they may stimulate minimal fish immune response; and 6) for practical application, the hormones are required at low dosages and therefore, economical to use (Zohar, 1989).



Peter, Chang, Nahorniak, Omeljaniuk, Sokolowska, Shih and Billard, (1986) demonstrated that gonadotropin releasing hormone in goldfish is inhibited by dopamine. The recent development of using pimozide (a dopamine antagonist), however, combined with either GnRHa or LHRHa, enhanced the efficiency of the hormone to induce ovulation. Pimozide blocks the action of dopamine, thus, a greater amount of GtHs is released from the pituitary gland to promote fish ovulation and spawning (Crim, 1982; Zohar, 1989). De leeuw Goos, Richter and Eding (1985) who studied various combinations and dosages of pimozide and LHRHa in a fixed ratio of 100:1, found that the limited effective dosage of the mixture was 5mg pimozide + 5 $\mu$ g LHRHa per kg fish.

Peter et al. (1988) suggested the use of the "Linpe technique", which is an effective technique to use GnRH agonist analogues (1 - 50  $\mu$ g of either LHRHa or sGnRHa) and dopamine antagonists (7  $\mu$ g - 5 mg of domperidone) for inducing of ovulation of cultured freshwater fish (Peter et al., 1993). This ovulation technique results in high ovulation rate, complete ovulation, short and predictable time to spawning, fertile eggs and no immune response to subsequent spawning of the same broodstock (Peter et al., 1993).

In addition, the efficacy of GnRHa may also depend on type of ovarian development. In fish with asynchronous ovarian development (i.e., multiple spawners), a single injection



may induce only partial ovulation and spawning (Zohar, 1989) and, therefore, the natural ovulation pattern must also be considered.

GnRHa and LHRHa are more effective than the native GnRH and LHRH, because they are resistant to enzymatic degradation. Thus, they have prolonged biological half lives (Goren, Zohar, Fridkin, Elhanati and Koch, 1990). For example, when GnRH or LHRH are injected to induce fish ovulation, the hormone induced GtHs rise rapidly and then disappear from circulation. In goldfish, the mGnRH disappeared within 12-60 mins (Sherwood and Harvey, 1986) and 1-2 hours in seabream (Goren, et al, 1990). Administration of these mGnRH and sGnRH may need to be repeated quite often in order to maintain GtH levels for approximately 48 hours. During this period, the GtH surge may be sufficient to induce ovulation in females that have not yet reached the final stages of vitellogenesis.

#### **7.1.3 Controlled Release of GnRH or LHRH in Teleosts**

To sustain high blood GtH levels, the hormone can be administered in the form of slow release implants. Hormones such as, LHRH and GnRH can be incorporated into pellets of either cholesterol, cellulose or silicone rubber (Sherwood, Crim, Carolsfeld and Walters, 1988; Crim, 1985). Such implants facilitate the slow release of GnRH and LHRH into the blood stream producing a constant GtH secretion from



the pituitary gland. Constant GtH release induces, accelerates and synchronizes spawning in a variety of fish species, such as rainbow trout (Crim, Evans and Vickerry, 1983), Atlantic salmon (Crim, Glebe and Scott, 1986), goldfish (Sokolowska, Peter, Nahorniak, Pan and Chang, 1984), milkfish (Lee, Tamaru, Banno and Kelley, 1986), sea bass (Harvey, Nacario, Crim, Juario and Marte, 1985) and seabream (Zohar, 1988).

In addition, there is a relationship between the method of administration and the rate of gonadal maturation (Funk and Donaldson, 1972). MacKinnon and Donaldson (1978) reported that multiple injections of SG-G100 three times / week ( $3 \mu\text{g/g}$  body weight/week) and a single SG-G100 in cholesterol pellet ( $9 \mu\text{g/g}$  body weight/3 week) resulted in similar GSI levels and accelerated the maturity of the gonads.

#### **7.1.4 Identification of Suitable Gonadal Stages in fish for Hormonal Therapy**

Timing of hormone administration in relation to gonadal development is an important factor in successful induce spawning of teleosts (Lam, 1982; 1985; Billard, 1989; Zohar, 1989). Generally, assessment of the gonadal stage is based on external characters of fish, such as the soft swollen abdomen and pink vent. The use of these criteria,



however, depend on personal experience and are often not precise enough to predict the right gonadal stage for hormonal administration (Lam, 1982). Therefore, to improve the efficacy of hormone therapy, it is recommended that the oocytes of the recipient females are biopsied to ensure and to select females at the correct gonadal stage for induced spawning treatment. By using either a polyethylene tube, glass canula or haematocrit capillary tube as an intraovarian catheter, the oocyte can be sampled. This technique, however, may not be suitable for some species such as tilapias which has a small genital papillae. Collecting the ovarian samples with any of the biopsy techniques may introduce additional stress and this may be a factor affecting successful spawning (Lam, 1982).

In addition to ovarian biopsies, the levels of some specific steroids could be used as an indicator of gonadal maturity. The maturation and ovulation of teleosts are associated with the production of steroid in the oocytes at the end of vitellogenesis (Nagahama, 1983; Fostire and Jalabert, 1986; Scott and Canario, 1987). Levels of T, GtHs and  $17\alpha,20\beta$ -P usually increase prior to ovulation. Thus, these hormone levels may be used to predict gonadal maturation of fishes. For example in coho salmon, *O. kisutch*, Fitzpatrick et al., (1987) used the levels of T to predict responsiveness of the females to the GnRHa treatments. Similarly in goldfish, the increase in T levels was noted to be an important physiological requirement for



the occurrence of the GtH surge (Kobayashi et al., 1986; 1988; 1989). In addition, the levels of total calcium ( $\text{Ca}^{2+}$ ) showed similar trend to T (chapters 5 and 6). Since the levels of T, GtHs,  $17\alpha,20\beta\text{-P}$  and  $\text{Ca}^{2+}$  are more easily measured without killing the fish, these levels may be useful indicators to determine the appropriate time for hormone induction (Donaldson, 1986).

#### 7.1.5 Hormonal Induction in Tilapias

Mass fry production is a major constraint in the intensive tilapia culture (Mires, 1982). Various hormones and neurotransmitter such as HCG, salmon GnRH or mammalian GnRH, GnRHa and the combination of GnRHa + pimozide have been used in order to synchronize the spawning of tilapias (Hyder, Shah and Kirschner, 1970; Hyder, Shah, Campbell and Dadzie, 1974; Babiker and Ibrahim, 1979b; Srisakultiew and Wee, 1988; King, Rivier, Vale and Millar, 1984; Gissis, Levavi-zermonsky, Bogomolnaya-Bass and Yaron, 1988; Roderick, Mair and Santiago, 1991) all of which have at best been partially successful in synchronizing spawning in tilapia species.

HCG is an effective agent in inducing *O. niloticus* spawning at a very high dose of 12,000 IU/kg whereas oestrone, oestriol, progesterone and pregnant mares serum (PMS) had all proved ineffective (Babiker and Ibrahim, 1979b). Similar studies with a lower dose of 250 and 500 IU/kg body



weight of HCG and pituitary hypophysation at the dose of 1 to 5 mg/kg body weight by Srisakultiew and Wee (1988) had also partially successful in the spawning induction of the same species (Srisakultiew and Wee, 1988).

Synthetic mammalian GnRH and salmon GnRH were found to be equally ineffective in stimulating testosterone (T) and oestradiol-17 $\beta$  (E<sub>2</sub>) release in *T.sparmanii* (King et al., 1984). These poor responses may be due to the endogenous hypothalamic GnRH of the fish being different to that of the salmon and mammals (King et al., 1984).

Effect of GnRHa (des-Gly<sup>10</sup> [D-Ala<sup>6</sup>]-ethylamide) with and without dopamine antagonist (reserpine or pimozide) on GtH release had been investigated in the female tilapia hybrid (*O. niloticus* x *O. aureus*) (Gissis et al., 1988). Their results have showed that neither pimozide or reserpine alone had any effect on GtH levels whereas GnRHa alone resulted in a moderate increase in GtH level.

The combination of GnRHa (100ug/kg) and pimozide (0.1 - 5.5mg/kg) resulted in a prolonged bimodal response of tilapia GtH (taGtH ; Gissis, 1988). Pimozide at the dose of 1.0 mg/kg body weight together with 100  $\mu$ g/kg body weight of GnRHa was less effective than the same GnRHa dose with 0.1 mg/kg pimozide (Gissis et al., 1988). They have additionally concluded that the GnRHa (des-Gly<sup>10</sup> [D-Ala<sup>6</sup>]) together with pimozide stimulated the secretion of GtH from



the pituitary of tilapia even at temperature as low as  $20 \pm 2^{\circ}\text{C}$ , which is below the normal requirement for reproductive development in tilapia. This dose (100  $\mu\text{g/kg}$  body weight of GnRHa + 0.1 mg/kg body weight of pimozide) was therefore selected for the hormone treatment in the present study.

In the study of Gissis, Levavi-Sivan, Rubin-Kedem, Ofir and Yaron, (1991), injection of the combination of super active GnRHa ([D-Ala<sup>6</sup>, Pro<sup>9</sup>-NET]-GnRH) 100  $\mu\text{g/kg}$  and reserpine 10 mg/kg fish body weight resulted in a bimodal pattern of taGtH. The first peak occurred at 30 minutes while a dramatic second peak was noted 6 hours after injection.

The efficiency of the dopamine antagonists used in hormonal induction may also vary. When three dopamine antagonists: metoclopramide (MET), pimozide (PIM) and domperidone (DOM) were compared at the same dose, all the these dopamine antagonists resulted in equipotent taGtH response within 3 hours (Gissis et al., 1991). Thereafter, only DOM treated fish showed a significant response to GnRHa at 6 hours, while at 12 and 24 hours pimozide was more effective than the other antagonists used (Gissis et al., 1991). When the fish were injected with a combination of the super active GnRHa (100  $\mu\text{g/kg}$  body weight) and metoclopramide (0.1 mg/kg body weight), the eggs from 9 out of 13 females ovulated and could be easily stripped within the period of 48 hours after injection, whereas no eggs were obtained by stripping



in the control group. In addition, the mean taGtH levels in these injected fish increased from 23 ng/ml to 469.3 ng/ml in the ovulated fish while in unovulated injected fish showed higher taGtH levels averaging 1,932 ng/ml (Gissis et al., 1991).

## 7.2 OBJECTIVES

To elucidate the problems associated with synchronised spawning for mass production of *O. niloticus* fry, the following trials were conducted:

- i. Two different methods of hormone administration were investigated. Fish were either injected with various doses of hormone mixture (experiments 1, 2 and 3) or implanted (experiment 4) with slow release pellets containing appropriate hormonal doses.
- ii. Following the results of the above trials the effect of hormone therapy of females at ten days post-spawning and social interaction on the efficacy of induced spawning was investigated (experiment 5).



## 7.3 METHODS

### 7.3.1 Effect of Different Methods of Hormone Administration on *O. niloticus* Spawning (Experiments 1, 2, 3 and 4)

#### 7.3.1.1 Experimental fish

*Oreochromis niloticus* broodstock were approximately 1 to 2 years old. The fish used for the injection (experiments 1, 2 and 3) and the implantation studies (experiment 4), were 60 - 155 g and 318 - 700 g body weight, respectively. The procedure for stocking, maintaining and feeding are described in chapter 6. The females with protruding genital papilla and swollen bellies were selected for hormonal applications. The fish were gently pressed on the sides of their abdomen to obtain oocyte samples (Babiker and Ibrahim, 1979b; Mires, 1982). If the extruded oocytes were golden yellowish in colour then they were selected for the study. The selected fish were anaesthetized in 2-phenoxy-ethanol (section 2.1.2) prior to hormonal injection and were subsequently stocked with males in 1 m<sup>2</sup> tanks or 2 m diameter spawning tanks at a sex ratio of 3 females : 1 male. Spawning results were observed daily over 2 week period after the first injection. Females which were carrying eggs in their mouths, were gently enticed into a net and the eggs were incubated to estimate their fertilisation rate as described for chapter 6.



#### 7.3.1.2 Hormonal injection

Des-Gly<sup>10</sup> [D-Ala<sup>6</sup>]-luteinizing hormone-releasing hormone ethylamide (LHRH; Sigma Chemical Company Ltd., Poole, Dorset, England) were dissolved and suspended in the vehicle solution consisting of 0.8% NaCl, 0.1% sodium metabisulphite and 0.25% bovine serum albumine (Richter et al., 1987). The final volume of the hormone mixture was made with the vehicle solution into 2 ml/kg of fish body weight. The fish were injected at the dosages of 10 to 300 µg/kg body weight and 0.1 mg/kg body weight pimozone (Sigma Chemical Company Ltd.). Anaesthetized fish were quickly injected at either 1, 2 or 3 times per week with the fixed total dose and amount of hormone solution. Detail of the three hormone injection experiments used are shown in Table 7.1.

Injections were administered into the peritoneal cavity at the base of the pectoral fin. The controls including the non-injected fish (untreated control) and those given vehicle-injection (placebo control) were compared with hormone treatments. The blood samples were drawn from the caudal vessels into heparinized 1 ml syringes just prior to hormonal treatment and at 3, 6, 12, 24 hours and at 3, 7, 10, 14 days post-injection. The blood samples were centrifuged and plasma samples were separated and stored at -20°C until required for total calcium analysis.



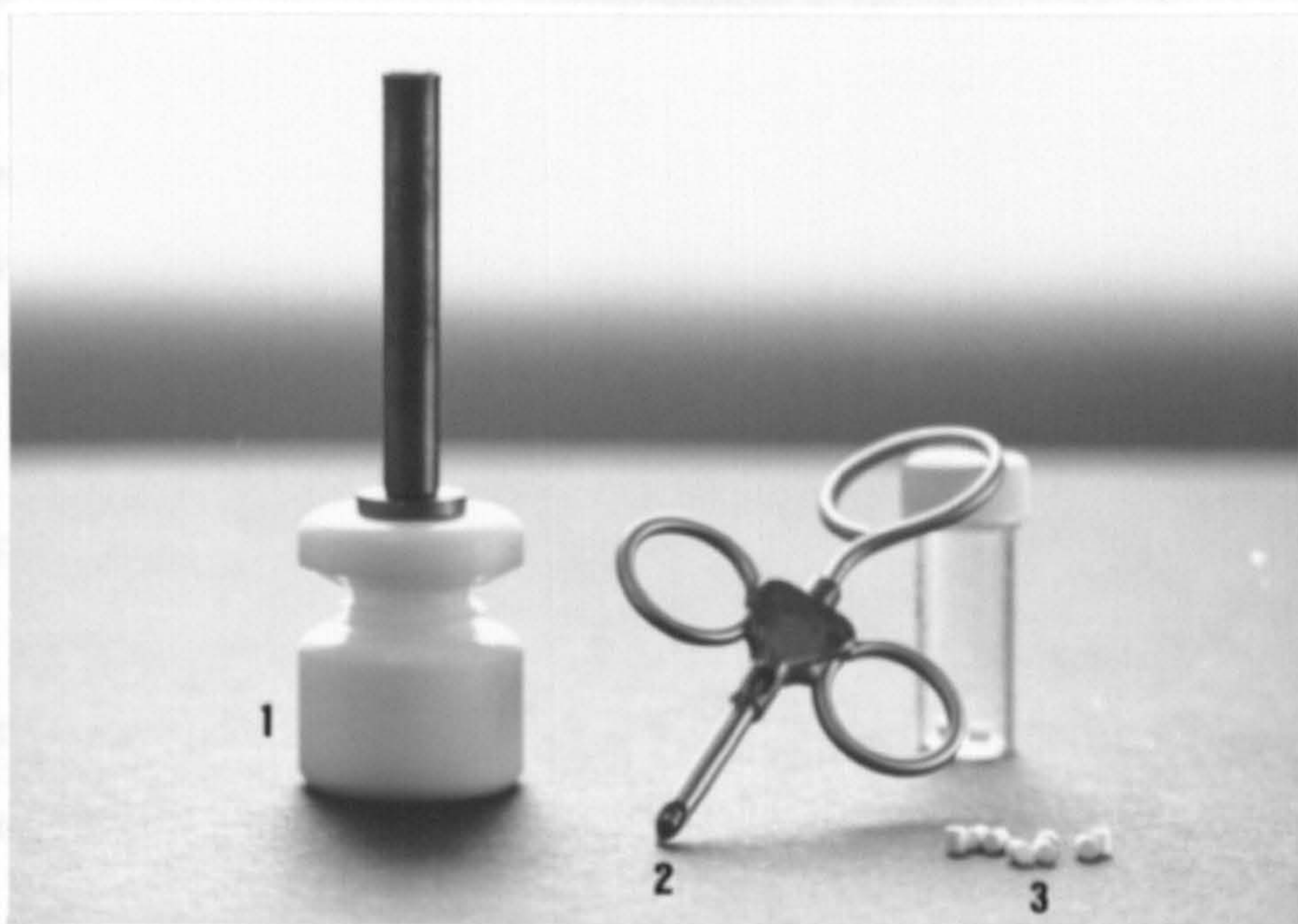


Plate 7.1: Illustration of equipment used for implanting the hormonal pellet. 1) a pellet maker, 2) an implanter, 3) 3mm compacted cholesterol and cellulose pellets.



#### 7.3.1.3 Hormonal pellet implantation (experiment 4)

The pellet maker and planter for making hormone pellets are shown in Plate 7.1. The hormone pellets containing approximately 50  $\mu$ g of LHRH (D-Ala<sup>6</sup>) were prepared by mixing the hormone with 30 mg of matrix. Two matrices were prepared according to Sherwood, Crim, Carolsfeld and Walters (1988). The first matrix which included 75% cholesterol (Sigma Ltd.) + 25% cellulose (Sigma Ltd.) and the second matrix contained 95% cholesterol + 5% cellulose (by weight). Thirty mg of hormone powdered matrices were then weighed and pressed into a cylindrical 3 mm pellet using a pellet maker (Parr Instrument Co., Moline, Ill.). The 95% cholesterol + 5% cellulose matrix tended to be brittle and fracture easily, therefore, the matrix was placed in a 30°C oven for an hour prior to being pressed.

The pellets were inserted into the muscle underneath the dorsal fin through a 0.5 cm planter (Parr Instrument Co.). The wound was sealed with tissue sealing antibiotic powder. The controls received either no-implantation (untreated control) or those given placebo pellets containing only cholesterol and cellulose (placebo control).



The fish were bled before implantation and after implantation at 6, 12, 24 hours and thereafter at bi-weekly intervals for 2 months. Spawning was observed daily as described in section 7.3.1.2. Plasma samples were stored at -20°C and subsequently analyzed for total calcium (section 2.4), testosterone (section 2.5) and tilapia gonadotropin II (taGtH) (analyzed by Dr. Yaron, Israel; according to the method of Bogomolnaya, Yaron, Hilge, Graesslin, Lichtenberg and Abraham, 1989).

#### 7.3.1.4 Histological studies

In order to evaluate the ovarian stages in the fish prior to the hormone administration, five fish were sacrificed for histological studies. Their length and weight were recorded and blood samples were taken from the caudal vein. The ovaries were dissected, weighed and fixed in Bouin's solution. Gonadosomatic index was calculated for each fish according to the protocol in section 2.1.4. The ovarian samples were processed in resin (section 2.2.1.1), cut into 5  $\mu\text{m}$  sections and stained with polychrome (section 2.2.1.2). The ovarian volume fraction of different oocyte stages were estimated according to section 2.3.



Table 7.1: Details of experimental design for the hormone treatments by the injection of LHRH + pimozide (Pi) and implantation of cholesterol and cellulose pellet experiments

| Treatments | Experiments   |  |  |  |
|------------|---|--|--|--|
|            | 1   | 2  | 3  | 4  |
| 1          | Untreated control                                       | Untreated control  | Untreated control  | Untreated control  |
| 2          | Placebo control   | Placebo control  | Placebo control  | Placebo control  |
| 3          | One injection/wk with each of 100µg LHRH + 0.1mgPi/kg   | One injection/wk with each of 300µg LHRH + 0.1mgPi/kg    | One injection/wk with each of 10µg LHRH + 0.1mgPi/kg     | A pellet of 75% cholesterol + 25% cellulose per 2 months |
| 4          | Two injections/wk with each of 50µg LHRH + 0.1mgPi/kg   | Two injections/wk with each of 150µg LHRH + 0.1mgPi/kg   | Two injections/wk with each of 5µg LHRH + 0.1mgPi/kg     | A pellet of 95% cholesterol + 5% cellulose per 2 months  |
| 5          | Three injections/wk with each of 33µg LHRH + 0.1mgPi/kg | Three injections/wk with each of 100µg LHRH + 0.1mgPi/kg | Three injections/wk with each of 3.3µg LHRH + 0.1mgPi/kg |  |
| 6          |   |  | One injection/wk with each of 100µg LHRH + 0.1mgPi/kg    |  |
| 7          |   |  | Two injections/wk with each of 50µg LHRH + 0.1mgPi/kg    |  |
| 8          |   |  | Three injections/wk with each of 33µg LHRH + 0.1mgPi/kg  |  |



7.2.2 Effect of the Hormonal Administration on 'Day 1' After Spawning and Social Interaction in the Spawning  
*Pragmocypris o. niloticus* (Experiment 5)

521 new yolk and at 12 h post spawning (12 h post spawning) they were held in 1 m<sup>2</sup> tanks. The first 24 h post spawning were held in 1 m<sup>2</sup> tanks. The first 24 h post spawning were held in 1 m<sup>2</sup> tanks.

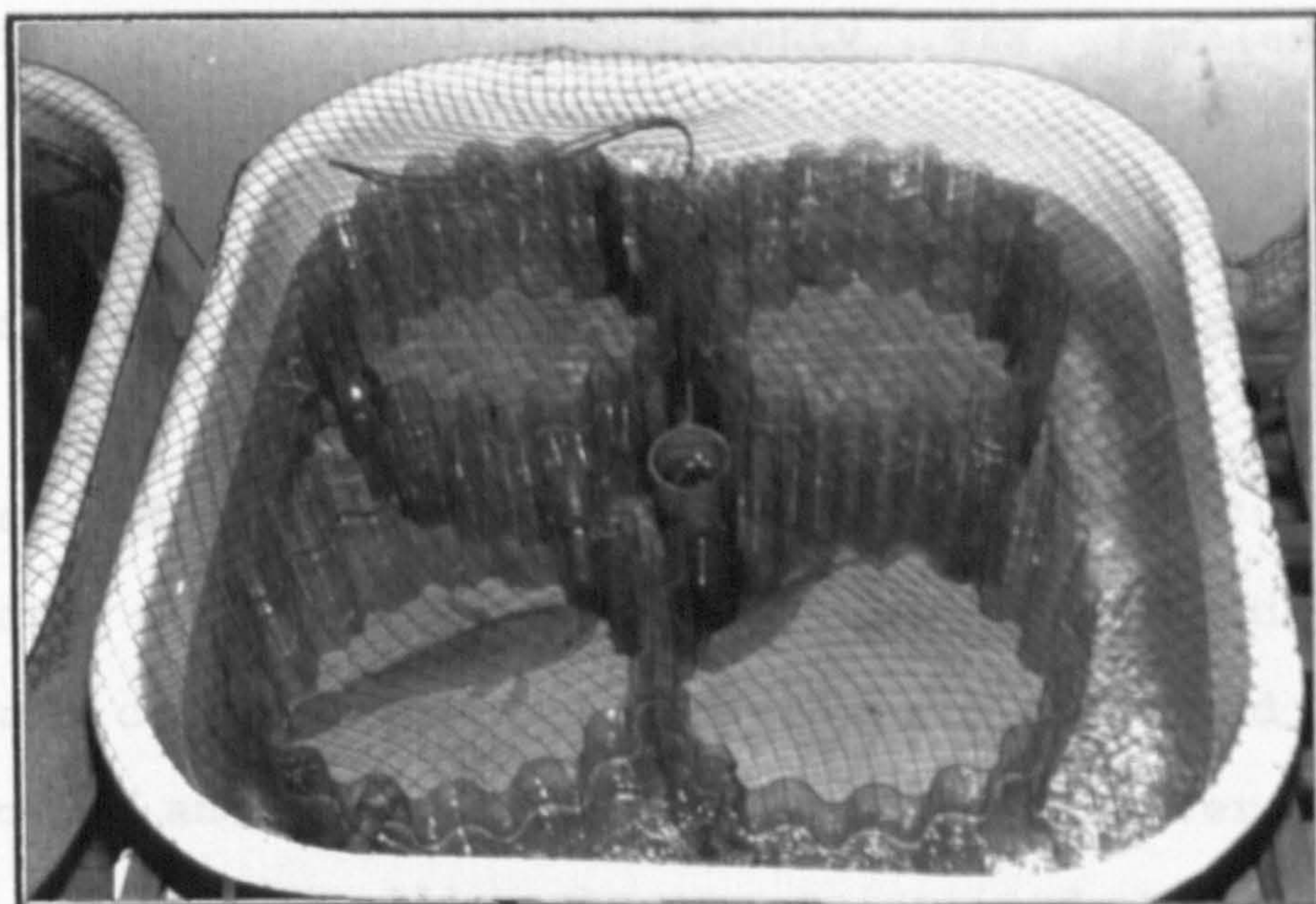


Plate 7.2 Illustration of the spawning tanks used for social 'limited contact' conditions in experiment 5. Four enclosed pens made from corrugated plastic sheets were placed in 1 m<sup>2</sup> tank for housing four individual females. One male was held in the same tank outside the pens.



### 7.3.2 Effect of the Hormone Administration on 'Day Ten After Spawning' and Social Interaction on the Spawning Frequency *O. niloticus* (Experiment 5)

*Oreochromis niloticus* females used in this study were 100 to 800 g in weight. They were held under two different spawning conditions. The first was described as an 'limited contact' condition in which only visual and chemical contacts were possible. Twenty six females were individually housed in separate transparent enclosed pens placed in 8 (1 m<sup>2</sup>) fibreglass tanks (Plate 7.2). A male was introduced into each tank but kept outside the pens.

The second condition was described as an 'unlimited contact' condition in which all visual, chemical and physical contacts were allowed. Eight females and 3 males were mixed and held together in two - 2 m diameter spawning tanks (Plate 6.1). All tanks were maintained at 27±1°C under a 12L:12D photoregime according to section 2.1. The fish were fed twice a day with the same commercial trout pellets No 4 (section 2.1.1).

The females housed under both conditions were observed daily and all spawnings recorded. When the females showed evidence of mouth brooding behaviour, the eggs were removed and numbers estimated. Eggs were incubated and fertilisation rate estimated (chapter 6). The egg numbers in each clutch was estimated using an indirect method



described by Rana (1986). The clutch of eggs was dried on absorbent paper and weighed on top pan balance (Mettler H100) to obtain total egg wet weight. A subsample of approximately 50 eggs was randomly sampled. The subsample eggs was counted, weighed and oven-dried over night at 50°C. The eggs were then cooled at room temperature in a desiccator and re-weighed. Total number of eggs, egg wet and dried weights were computed as these following formula:

$$\text{Total No of egg} = \frac{\text{subsampled egg No}}{\text{subsampled egg wet weight (mg)}} \times \text{Total egg wet weight (mg)}$$

$$\text{Mean egg wet weight (mg)} = \frac{\text{subsampled egg wet weight (mg)}}{\text{subsampled egg No.}}$$

$$\text{Egg dry weight (mg)} = \frac{\text{subsampled egg dried weight (mg)}}{\text{subsampled egg No.}}$$

Either two ml/kg female body weight dilution of the hormone combination (100 µg LHRH + 0.1 mg pimozone) or two ml/kg female body weight of vehicle solution (according to section 7.3.1.2) was injected into the intraperitoneal cavity of the females at days 10 - 12 after spawning. After the females had spawned, the same doses were repeated at 10 day interval for 2 to 3 spawning cycles of each female. Spawning day, egg numbers, egg weight and fertilisation rate of the fish were individually recorded.



## 7.4 RESULTS

### 7.4.1 Effect of Different Methods of Hormone Administration on *O. niloticus* Spawning (Experiments 1, 2, 3 and 4)

#### 7.4.1.1 Gonadal stage of ovaries prior to the hormonal administration

##### a. Gonadosomatic index (GSI)

At the beginning of each trial, five fish from each experiment were sacrificed to estimate the gonadal stages of the fish prior to the treatments. Non-parametric analysis indicated that the GSI of females in experiment 1 (1.93%) was significantly ( $P < 0.05$ ) lower than that in experiment 2 (GSI = 2.59%) whereas GSIs in the other experiments showed no significant ( $P > 0.05$ ) differences (Table 7.2).

##### b. Ovarian volume fractions of the ovaries

The different oocyte stages in the ovaries were quantified prior to hormonal treatments (Table 7.2). Ovaries of females used in experiment 1 contained a significantly ( $P < 0.05$ ) higher proportion of stage 2 oocytes and this result corresponded to their lowest female GSI (Table 7.2).



The volume fraction of stage 6 oocytes (59.4, 65.2 and 55.6% in experiment 1, 2 and 3, respectively) prior to hormonal treatments showed no statistical difference ( $P>0.05$ ) between the three injection experiments but was significantly ( $P<0.05$ ) lower than that of the implantation experiment (70.5%; Table 7.2). In addition, although the mean GSI (2.67%) of the females used in experiment 3 was the highest, their ovaries contained a significantly ( $P<0.05$ ) higher proportion of atretic oocytes (24.47% in the ovary).

Table 7.2: Initial volume fraction of the different stages of *O. niloticus* oocytes before LHRH administration by injection and implantation

| Expt  | Percentages of the volume fraction of oocyte in different stages (mean $\pm$ SE) |                                 |                                 |                                 |                                  |                                  |
|---|--|---------------------------------|---------------------------------|---------------------------------|----------------------------------|----------------------------------|
|   | stage 2  | stage 3                         | stage 4                         | stage 5                         | stage 6                          | atresia                          |
| 1.<br>GSI=1.93 <sup>b</sup><br>$\pm 1.56\%$ | 5.10 <sup>b</sup><br>$\pm 0.58$  | 6.03 <sup>b</sup><br>$\pm 1.01$ | 4.27 <sup>a</sup><br>$\pm 1.12$ | 6.06 <sup>a</sup><br>$\pm 1.54$ | 59.37 <sup>a</sup><br>$\pm 3.46$ | 8.97 <sup>a</sup><br>$\pm 1.94$  |
| 2.<br>GSI=2.59 <sup>a</sup><br>$\pm 1.78\%$ | 3.24 <sup>a</sup><br>$\pm 0.61$  | 3.60 <sup>a</sup><br>$\pm 0.83$ | 1.73 <sup>a</sup><br>$\pm 0.67$ | 5.60 <sup>a</sup><br>$\pm 1.74$ | 65.20 <sup>a</sup><br>$\pm 4.08$ | 11.16 <sup>a</sup><br>$\pm 2.61$ |
| 3.<br>GSI=2.69 <sup>a</sup><br>$\pm 0.88\%$ | 2.23 <sup>a</sup><br>$\pm 0.39$  | 3.43 <sup>a</sup><br>$\pm 0.65$ | 2.13 <sup>a</sup><br>$\pm 0.71$ | 6.30 <sup>a</sup><br>$\pm 1.65$ | 55.63 <sup>a</sup><br>$\pm 3.90$ | 24.47 <sup>b</sup><br>$\pm 3.68$ |
| 4.<br>GSI=2.17 <sup>a</sup><br>$\pm 0.49\%$ | 2.04 <sup>a</sup><br>$\pm 0.45$  | 4.22 <sup>a</sup><br>$\pm 0.93$ | 4.40 <sup>a</sup><br>$\pm 1.74$ | 8.93 <sup>a</sup><br>$\pm 1.93$ | 70.47 <sup>b</sup><br>$\pm 3.53$ | 5.11 <sup>a</sup><br>$\pm 2.10$  |

The same superscripts within columns indicate no significant ( $P>0.05$ ) differences.



#### 7.4.1.2 Effect of hormonal injection and doses on induced spawning

##### a. Experiment 1

The combination of 100 $\mu$ g LHRH + 0.1mg pimozide administered as either 1, 2 or 3 injections per week was ineffective in inducing spawning within a 2 week period. The females given the placebo spawned more frequently than those in the hormone treated and untreated control fish (Table 7.3).

##### b. Experiment 2

In this trial the dosage of LHRH was increased to 300 $\mu$ g/kg body weight with the same amount of pimozide. More fish spawned than those in experiment 1. This may be due to their more advanced gonadal development (higher GSI = 2.59%) and the higher proportion of stage 6 oocytes (65.2%). In the first week of the experiment, 50% (4 from 8 fish) of the females in the placebo control group and the 3 injections / week treatment, followed by 37.5% (3 from 8 fish) of the females in the 2 injections / week treatment spawned. Lower spawning frequency was found in the untreated control group and the 1 injection / week treatment (Table 7.3). The eggs obtained from this experiment, however, were unfertilised (Table 7.3).



### c. Experiment 3

In order to resolve the effect of too high dose on the fertilisation rate of the eggs observed in experiment 2 (Table 7.2), two lower dosages of: 10  $\mu\text{g}$  and 100  $\mu\text{g/kg}$  LHRH + 0.1mg/kg pimozide were used in experiment 3. The spawning results in the first week showed that only one fish spawned from 8 fish in each treatment including the controls and no spawning was observed in the second week (Table 7.3).



Table 7.3: Effect of LHRH + pimozide injections on the spawning and fertilisation rate of *O. niloticus* in experiments 1, 2 and 3

| Expt | Dosages<br>(LHRH +<br>pimozide<br>per kg<br>fish) | Treatments<br>(per week) | Pre -<br>treat-<br>ment GSI | Ca <sup>++</sup><br>(mg/l) | Number of fish<br>spawned / total<br>fish used<br>(% spawned) |              | Average<br>fertilisation of<br>egg (%) |           |
|------|---|--------------------------|-----------------------------|----------------------------|---|--------------|--|-----------|
|      |   |                          | mean<br>±SE                 | mean<br>±SE                | 1st<br>wk   | 2nd<br>wk    | 1st<br>wk                              | 2nd<br>wk |
| 1    | 100µg<br>+0.1mg                                   | 1) control<br>untreated  | 1.93<br>±1.56<br>(n=5)      | 23.05<br>±2.64<br>(n=5)    | 1/5<br>(20%)  | -            | 3.36%                                  | -         |
|      |   | 2) control<br>vehicle    |                             |                            | -   | 3/6<br>(50%) | -                                      | 11.46     |
|      |   | 3) 1 injection           |                             |                            | -   | 1/6<br>(17%) | -                                      | 9.43      |
|      |   | 4) 2 injections          |                             |                            | -   | -            | -                                      | -         |
|      |   | 5) 3 injections          |                             |                            | -   | 1/6<br>(17%) | -                                      | 0         |
| 2    | 300µg +<br>0.1mg                                  | 1) control<br>untreated  | 2.59<br>±1.78<br>(n=4)      | 34.49<br>±6.83<br>(n=4)    | 2/8<br>(25%)  | -            | 0                                      | -         |
|      |   | 2) control<br>vehicle    |                             |                            | 4/8<br>(50%)  | -            | 0                                      | -         |
|      |   | 3) 1 injection           |                             |                            | 1/8<br>(13%)  | 1/8<br>(13%) | 1.84                                   | 0         |
|      |   | 4) 2 injections          |                             |                            | 3/8<br>(38%)  | -            | 2.8                                    | 0         |
|      |   | 5) 3 injections          |                             |                            | 4/8<br>(50%)  | -            | 0                                      | -         |
| 3    | 10 µg +<br>0.1mg                                  | 1) control<br>untreated  | 2.69<br>±0.88<br>(n=5)      | 26.98<br>±4.19<br>(n=5)    | 0   | 0            | -                                      | -         |
|      |   | 2) control<br>vehicle    |                             |                            | 1/8<br>(13%)  | 0            | 0                                      | -         |
|      |   | 3) 1 injection           |                             |                            | 0   | 0            | -                                      | -         |
|      | 100 µg +<br>0.1 mg                                | 4) 2 injections          |                             |                            | 1/8<br>(13%)  | 0            | 39.35                                  | -         |
|      |   | 5) 3 injections          |                             |                            | 1/8<br>(13%)  | 0            | 0                                      | -         |
|      |   | 6) 1 injection           |                             |                            | 0<br>(13%)  | 0            | -                                      | -         |
|      |   | 7) 2 injections          |                             |                            | 1/8<br>(13%)  | 0            | 2.32                                   | -         |
|      |   | 8) 3 injections          |                             |                            | 1/8<br>(13%)  | 0            | 4.8                                    | -         |



#### 7.4.1.3. Effect of hormonal pellet implantation on spawning frequency of females (Experiment 4)

The females selected for hormone implant studies contained the highest proportion of stage 6 oocytes when compared to those in the hormone injections (Table 7.2). The implanted fish, however, showed a negative response to both fast and slow (75 and 95% cholesterol, respectively) releasing hormone pellets. All of the controls, however, showed regular spawning over the period of two months (Table 7.5). To elucidate the effects of hormone treatments on induced spawning, total calcium ( $\text{Ca}^{2+}$ ), testosterone (T) and gonadotropin (taGtH) in plasma samples were analyzed. The plasma levels of  $\text{Ca}^{2+}$ , T and taGtH are presented in Figure 7.2. At the beginning of the treatment, these were 24.23 mg%, 51.83 and 17.43 ng/ml, respectively. After implantation, the  $\text{Ca}^{2+}$  levels of the females implanted with the 75 and 95% cholesterol pellets showed the same trends as the controls until day 38 post-implant; when the  $\text{Ca}^{2+}$  levels slightly increased from those controls and peaked at day 45 post-implant (one fish spawned in 75% cholesterol pellet).



Table 7.4: Ovarian volume fractions of *O. niloticus* 8 weeks after  
LHRH implantation (Experiment 4)

| Treatments                                   | Percentage of the volume fractions of oocytes in<br>different stages (mean $\pm$ SE) |                                 |                                 |                                 |                                  |                                  |
|--|--|---------------------------------|---------------------------------|---------------------------------|----------------------------------|----------------------------------|
|  | stage<br>2   | stage<br>3                      | stage<br>4                      | stage<br>5                      | stage<br>6                       | atresia                          |
| Untreated<br>control<br>GSI=3.77 $\pm$ 1.19% | 2.20 <sup>a</sup><br>$\pm$ 0.75  | 2.93 <sup>a</sup><br>$\pm$ 1.41 | 2.67 <sup>a</sup><br>$\pm$ 1.93 | 7.33 <sup>a</sup><br>$\pm$ 2.11 | 60.09 <sup>a</sup><br>$\pm$ 4.37 | 18.93 <sup>b</sup><br>$\pm$ 3.78 |
| Placebo control<br>GSI=2.77 $\pm$ 0.43%      | 2.03 <sup>a</sup><br>$\pm$ 0.44  | 3.93 <sup>a</sup><br>$\pm$ 0.81 | 4.18 <sup>a</sup><br>$\pm$ 0.11 | 8.59 <sup>a</sup><br>$\pm$ 2.07 | 38.8 <sup>b</sup><br>$\pm$ 3.63  | 33.42 <sup>b</sup><br>$\pm$ 5.07 |
| 75% Cholesterol<br>GSI=3.05 $\pm$ 0.51%      | 1.67 <sup>a</sup><br>$\pm$ 0.39  | 3.07 <sup>a</sup><br>$\pm$ 0.65 | 2.18 <sup>a</sup><br>$\pm$ 0.82 | 4.70 <sup>a</sup><br>$\pm$ 1.21 | 67.73 <sup>a</sup><br>$\pm$ 3.0  | 14.0 <sup>a</sup><br>$\pm$ 2.80  |
| 95% Cholesterol<br>GSI=4.11 $\pm$ 0.65%      | 1.57 <sup>a</sup><br>$\pm$ 0.41  | 1.29 <sup>a</sup><br>$\pm$ 0.57 | 1.56 <sup>a</sup><br>$\pm$ 0.69 | 4.50 <sup>a</sup><br>$\pm$ 1.62 | 71.33 <sup>a</sup><br>$\pm$ 3.22 | 11.07 <sup>a</sup><br>$\pm$ 2.29 |

The same superscripts within columns indicate no significant ( $P>0.05$ ) differences.

Table 7.5: Numbers of fish spawned in each week and fertilisation rate of  
eggs in the LHRH implantation experiment (Experiment 4)

| Wks            | Treatments           |             |                 |             |                    |             |                    |             |
|----------------|----------------------|-------------|-----------------|-------------|--------------------|-------------|--------------------|-------------|
|                | Untreated<br>control |             | Sham<br>control |             | 75%<br>cholesterol |             | 95%<br>cholesterol |             |
|                | Spawn<br>(%)         | Fert<br>(%) | Spawn<br>(%)    | Fert<br>(%) | Spawn<br>(%)       | Fert<br>(%) | Spawn<br>(%)       | Fert<br>(%) |
| 0              | -                    | -           | -               | -           | -                  | -           | -                  | -           |
| 1              | 12.5                 | 89.8        | 22.2            | 32.5        | 11.1               | 0           | 0                  | -           |
| 2              | 25                   | 64.5        | 0               | -           | 11.1               | 34.4        | 0                  | -           |
| 4              | 0                    | -           | 28.5            | 75.3        | 0                  | -           | 0                  | -           |
| 6              | 12.5                 | 0           | 42.9            | 78.4        | 0                  | -           | 0                  | -           |
| 8              | 12.5                 | 20.5        | 57.1            | 71.4        | 12.5               | 6.8         | 0                  | -           |
| Total<br>spawn | 50                   | 59.8        | 71.4            | 64.4        | 37.4               | 13.7        | 0                  | -           |



Testosterone levels of females implanted with the 75 and 95% cholesterol pellets maintained at their initial high levels for 6 hours then dropped at day one post-implant. These low levels of T were maintained until day 45 post-implant, when the levels began to increase and peaked higher than the controls at day 55 post-implant no spawnings were observed in females implanted with 95% cholesterol pellets over the 2 months period.

The GtH levels of the females given the 75 and 95% cholesterol pellets also increased 6 hours post-implant. The GtH levels of the 75% cholesterol pellet maintained similar low levels until day 45, when the levels showed a dramatic increase (one fish spawned) and dropped at the end of the experiment. In 95% cholesterol pellet group, the GtH levels peaked only once at 6 hours post-implant and the levels were maintained at the similar low levels as the control groups throughout the experiment. Whereas the untreated and placebo controls females showed a regular spawning (Table 7.5). The GtH levels of the controls did not show any peak over the 2 month period.



Figure 7.2 Effect of 75% and 95% cholesterol pellets containing 50 $\mu$ g LHRH per pellet (average 100  $\mu$ g LHRH/kg body) on blood hormonal levels of *O. niloticus* females:

- (a) average levels of total calcium
- (b) average levels of testosterone
- (c) average levels of gonadotropin II (taGtH)



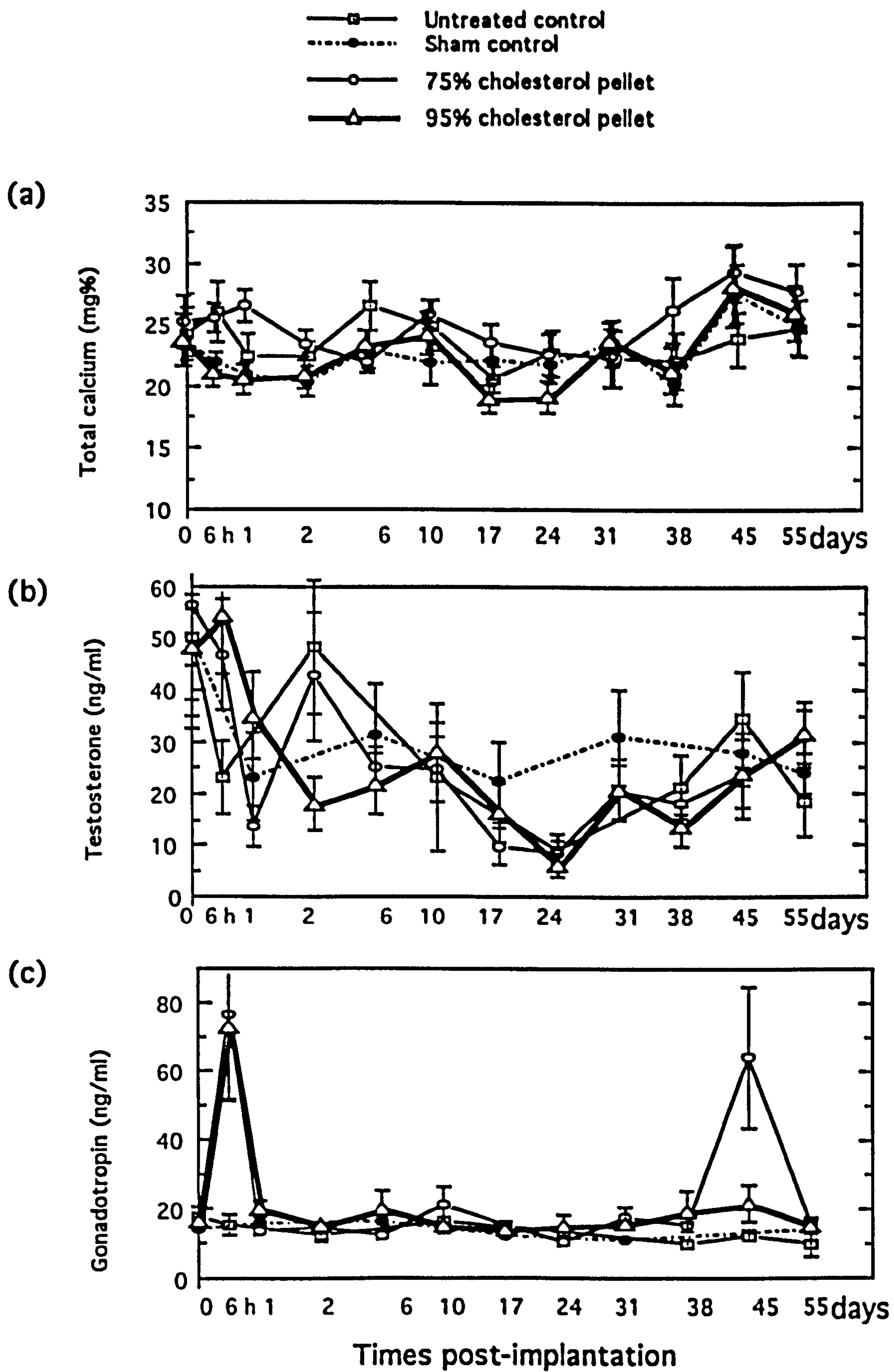


Figure 7.2



#### 7.4.2 Effect of the Hormone Administration on 'Day Ten After Spawning' and Social Interaction on the Spawning Frequency of *O. niloticus* (Experiment 5)

##### 7.4.2.1. 'Limited contact' condition

In this trial, visual but no physical contact was allowed between the male and the females. The combination of the hormone injection under this condition induced the females to spawn at the median time of 2, 6 and 3 days post-injection in the first, second and third cycles, respectively. The placebo control on the other hand, spawned at the median of 7 days post-injection in the first and second cycles and 8 days post-injection in the third cycle (Figure 7.3). The spawning period of each cycle of the hormone treated fish was significantly ( $P < 0.05$ ) shorter than that of the placebo control. The hormone treatment therefore, accelerated the spawning by 1 to 5 days earlier when compared with the control (Figure 7.3; Table 7.6).

Within 4 days post-injection 47 - 86% and 8 - 22% females of the hormone treated group and the placebo control group, respectively, spawned. The hormone treatment caused all the fish to spawn within 7 to 14 days post-injection while all the control fish spawned within 13 to 32 days post-injection (Figure 7.3).



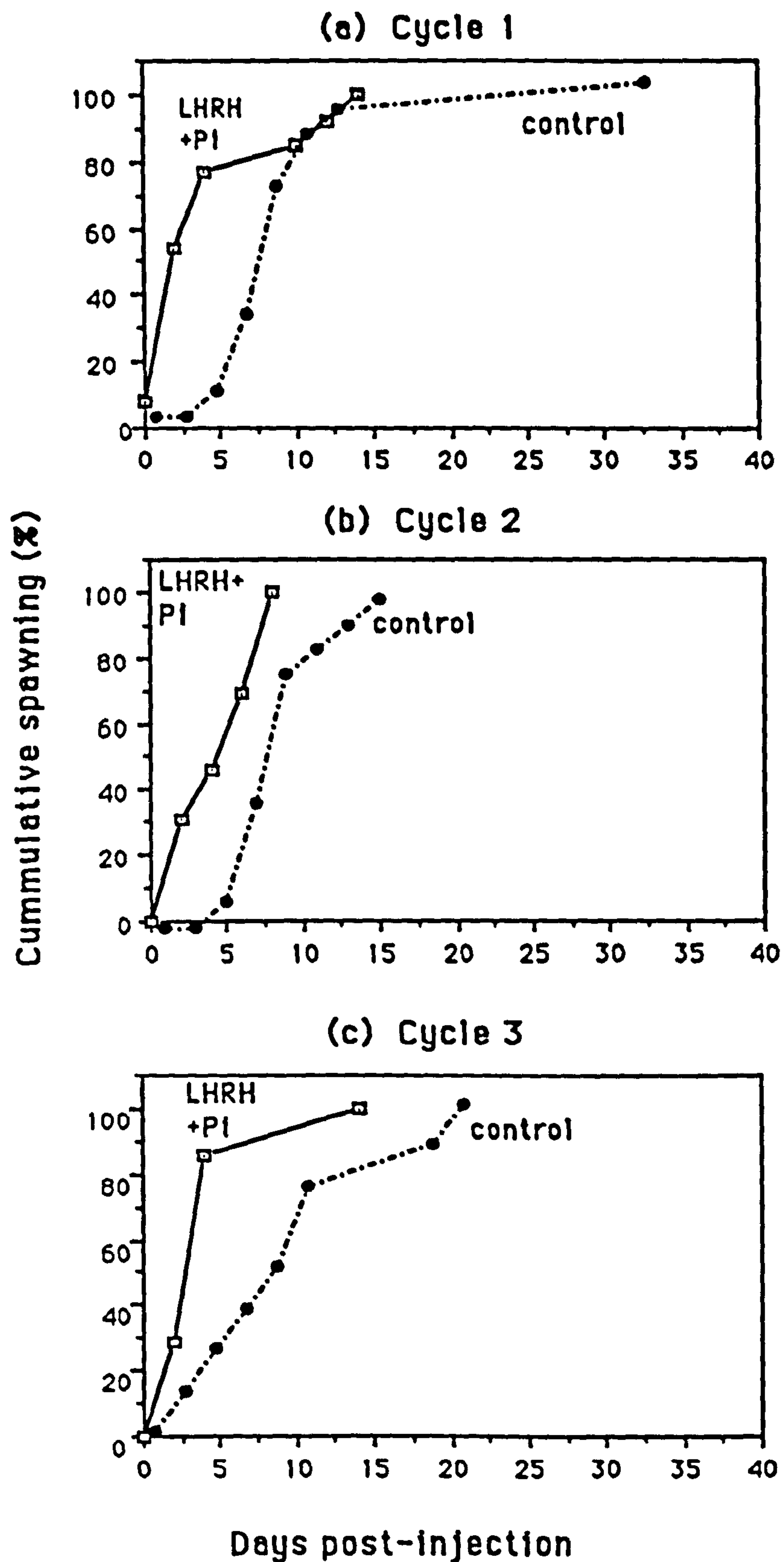


Figure 7.3 Cumulative spawning of *O. niloticus* after the treatment with LHRH + pimozide on 'day ten after spawning'. The fish were kept under the 'limited contact' condition. (a) Spawning cycle 1; (b) Spawning cycle 2; (c) Spawning cycle 3.



Table 7.6: Effect of 100µg LHRH/kg body weight + 0.1mg/kg body weight of pimozide administered to *O. niloticus* female at 'day ten after spawning' on spawning period under two different conditions.

| Conditions            | C<br>y<br>c<br>l<br>e | Placebo control    |                 |     |     | LHRH + Pimozide   |                 |     |     |
|-----------------------|-----------------------|--------------------|-----------------|-----|-----|-------------------|-----------------|-----|-----|
|                       |                       | mean±SE            | median          | min | max | mean±SE           | median          | min | max |
| 1. Limited contact    | 1                     | 9.0±2.02<br>(n=13) | 7 <sup>a</sup>  | 3   | 32  | 4.2±1.3<br>(n=13) | 2 <sup>b</sup>  | 0   | 14  |
|                       | 2                     | 7.5±0.6<br>(n=13)  | 7 <sup>a</sup>  | 4   | 13  | 4.0±0.7<br>(n=12) | 6 <sup>b</sup>  | 1   | 7   |
|                       | 3                     | 9.6±1.9<br>(n=9)   | 8 <sup>a</sup>  | 2   | 19  | 4.1±1.6<br>(n=7)  | 3 <sup>b</sup>  | 1   | 13  |
| 2. Un-limited contact | 1                     | 15.3±5.5<br>(n=8)  | 10 <sup>a</sup> | 0   | 40  | 14.1±4.3<br>(n=8) | 10 <sup>a</sup> | 4   | 34  |
|                       | 2                     | 18.8±6.5<br>(n=6)  | 19 <sup>a</sup> | 4   | 40  | 16.2±4.8<br>(n=5) | 12 <sup>a</sup> | 8   | 35  |

The same superscripts within the same columns indicate no significant ( $P>0.05$ ) differences.

Table 7.7: Spawning history of female no. 834, relative fecundity and fertilisation rate of *O. niloticus* eggs on spawning cycles before and after injection of 100µg/kg body weight of LHRH + 0.1mg/kg body weight of pimozide under the 'limited contact' condition.

| Treatments    | Spawning no. | Spawning cycle (days) | Relative fecundity (eggs/kg) | Egg dry weight mean±SE (mg) | Fertilisation (%) |
|---------------|--------------|-----------------------|------------------------------|-----------------------------|-------------------|
| Before        | 1            | -                     | 2380                         | -                           | -                 |
|               | 2            | 42 <sup>a</sup>       | 1609                         | -                           | -                 |
| LHRH+pimozide | 3            | 10+2 <sup>b</sup>     | 3803                         | -                           | -                 |
| [100µg/kg]    | 4            | 10+2 <sup>b</sup>     | 3079                         | -                           | -                 |
| +             | 5            | 10+1 <sup>b</sup>     | 3374                         | -                           | 88                |
| [0.1mg/kg]    | 6            | 10+2 <sup>b</sup>     | 2603                         | 2.84                        | 100               |
|               | 7            | 15+1 <sup>b</sup>     | 3498                         | 2.84                        | 89.9              |

<sup>a</sup> Indicates spawning cycle period in that cycle.

<sup>b</sup> Indicates luteolytic period (days) + spawning period (days post-injection) = spawning cycle.



#### 7.4.2.2 The 'Unlimited contact' condition

In this condition the males and females had unlimited visual, chemical and physical contact. Hormone treatment induced the fish to spawn at the median time of 10 to 12 days post-injection in the first and second cycle, respectively. Whereas the control fish spawned at 10 to 19 days post-injection. The hormone treatment under this condition was ineffective ( $P>0.05$ ) in accelerating the spawning period of the fish (Figure 7.4).

Within 12 days post-injection 60 - 62% and 40 - 65% of the females in the hormone treated group and the placebo control group, respectively, spawned (Figure 7.4). All the females spawned within the period of 35 - 40 days and 40 days post-injection of the hormone treated group and the placebo control group, respectively. The hormone treatment group under this condition had partially accelerated the spawning period of these females.



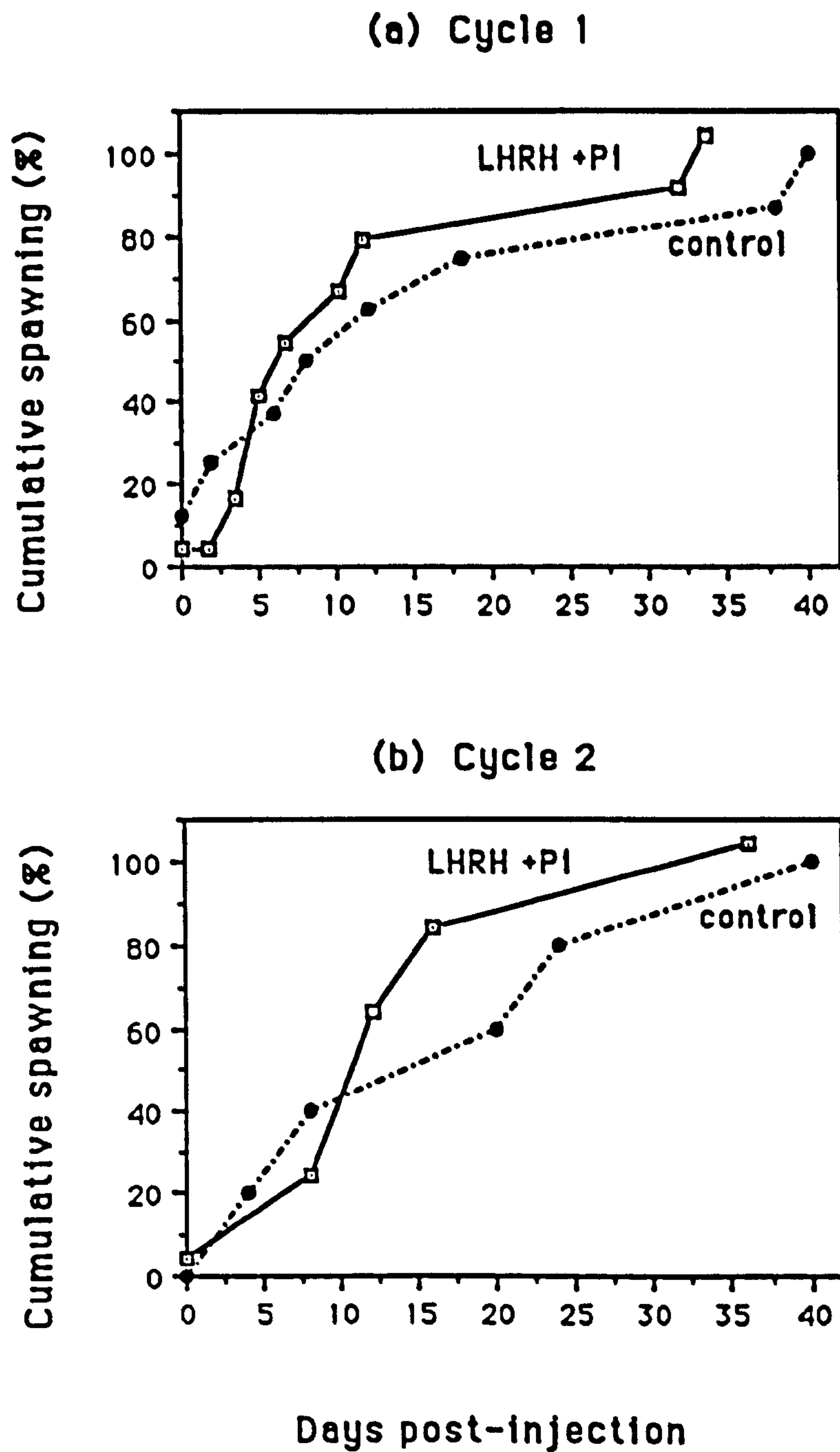


Figure 7.4: Cumulative spawning of *O. niloticus* after the treatment of LHRH + pimozone at 'day ten post-spawning'. The fish were kept under the 'unlimited contact' condition. (a) Spawning cycle 1; (b) Spawning cycle 2.



#### 7.4.2.3 Effect of hormone on the quantity and quality of *O. niloticus* eggs

In the 'unlimited contact' trial, the relative fecundities of the fish in the second, third and fourth spawning cycles were significantly ( $P < 0.05$ ) greater than those numbers in the first spawning cycle. In addition, there was no significant difference in fecundity ( $P > 0.05$ ) between the hormone treated and the placebo control groups and between spawning cycles (Table 7.8; Figure 7.5a).

The fish under 'unlimited contact' condition were allowed to spawn naturally. Thus, the fertilisation rates were easily obtained. The fertilisation rates of eggs between the hormone treatment and the placebo control groups within and between spawning cycles were not statistically ( $P > 0.05$ ) different (Figure 7.6).

The fertilisation rates of egg clutches from the females in the 'limited contact' condition were difficult to obtain. It was not possible to observe the females clearly in the fibre glass tanks and therefore stripping the eggs at the right time proved difficult. Eventually, some data was recorded from female no. 834 which spawned 7 times. The eggs from the last three spawning cycles were manually stripped, fertilised and artificially incubated. The fertilisation rates of these eggs ranged from 88 to 100% (Table 7.7).



Hormone treatment and social interaction affected egg quality of the fish. The LHRH treatment group in the 'limited contact' condition resulted in significantly ( $P < 0.05$ ) smaller egg size when compared with the placebo control (Figure 7.7). In contrast, there was no significant ( $P > 0.05$ ) difference in the egg size between all females held under the 'unlimited contact' condition.



Table 7.8: Quantity and quality of *O. niloticus* eggs from females treated with 100µgLHRH/kg body weight + 0.1 mg/kg of body weight pimozone under two different spawning conditions.

| Conditions            | C<br>y<br>c<br>l<br>e | Relative fecundity egg/kg (mean±SE)  |                                      | Egg wet weight mean±SE (mg)          |                                      | Egg dry weight mean±SE (mg)          |                                      |
|-----------------------|-----------------------|--------------------------------------|--------------------------------------|--------------------------------------|--------------------------------------|--------------------------------------|--------------------------------------|
|                       |                       | Control                              | LHRH+<br>Pi                          | Control                              | LHRH+<br>Pi                          | Control                              | LHRH+<br>Pi                          |
| 1. Limited contact    | 1                     | 3358 <sup>a1</sup><br>±345<br>(n=13) | 3968 <sup>a1</sup><br>±263<br>(n=13) | 7.2 <sup>a1</sup><br>±0.83<br>(n=5)  | 7.3 <sup>a1</sup><br>±0.68<br>(n=4)  | 3.4 <sup>a1</sup><br>±0.15<br>(n=5)  | 3.0 <sup>a1</sup><br>±0.16<br>(n=4)  |
|                       | 2                     | 4236 <sup>b1</sup><br>±227<br>(n=13) | 5241 <sup>b1</sup><br>±503<br>(n=13) | 8.0 <sup>a1</sup><br>±0.71<br>(n=11) | 6.3 <sup>a2</sup><br>±0.31<br>(n=9)  | 3.3 <sup>a1</sup><br>±0.09<br>(n=11) | 2.5 <sup>a2</sup><br>±0.16<br>(n=9)  |
|                       | 3                     | 5436 <sup>b1</sup><br>±311<br>(n=13) | 5159 <sup>b1</sup><br>±558<br>(n=12) | 7.3 <sup>a1</sup><br>±2.37<br>(n=13) | 6.7 <sup>a1</sup><br>±0.29<br>(n=11) | 3.1 <sup>a1</sup><br>±0.10<br>(n=13) | 2.8 <sup>a2</sup><br>±0.12<br>(n=11) |
|                       | 4                     | 5502 <sup>b1</sup><br>±532<br>(n=9)  | 6353 <sup>b1</sup><br>±560<br>(n=7)  | 7.4 <sup>a1</sup><br>±0.33<br>(n=12) | 6.1 <sup>a2</sup><br>±0.17<br>(n=10) | 3.1 <sup>a1</sup><br>±0.14<br>(n=12) | 2.5 <sup>a2</sup><br>±0.09<br>(n=10) |
| 2. Un-limited contact | 1                     | 2979 <sup>a1</sup><br>±423<br>(n=8)  | 3079 <sup>a1</sup><br>±432<br>(n=8)  | 7.7 <sup>a1</sup><br>±0.64<br>(n=8)  | 8.4 <sup>a1</sup><br>±0.56<br>(n=8)  | 3.1 <sup>a1</sup><br>±0.26<br>(n=8)  | 3.4 <sup>a1</sup><br>±0.19<br>(n=8)  |
|                       | 2                     | 3143 <sup>a1</sup><br>±942<br>(n=7)  | 3930 <sup>a1</sup><br>±758<br>(n=6)  | 7.4 <sup>a1</sup><br>±0.48<br>(n=7)  | 7.8 <sup>a1</sup><br>±0.38<br>(n=6)  | 3.9 <sup>a1</sup><br>±0.80<br>(n=7)  | 3.3 <sup>a1</sup><br>±0.13<br>(n=6)  |
|                       | 3                     | 4690 <sup>a1</sup><br>±1763<br>(n=3) | 3946 <sup>a1</sup><br>±1066<br>(n=4) | 6.9 <sup>a1</sup><br>±0.71<br>(n=3)  | 8.1 <sup>a1</sup><br>±0.41<br>(n=4)  | 2.9 <sup>a1</sup><br>±0.29<br>(n=3)  | 3.5 <sup>a1</sup><br>±0.29<br>(n=4)  |

Means with the same letters and numbers within the same columns and row, respectively, indicate no significant ( $P>0.05$ ) differences.



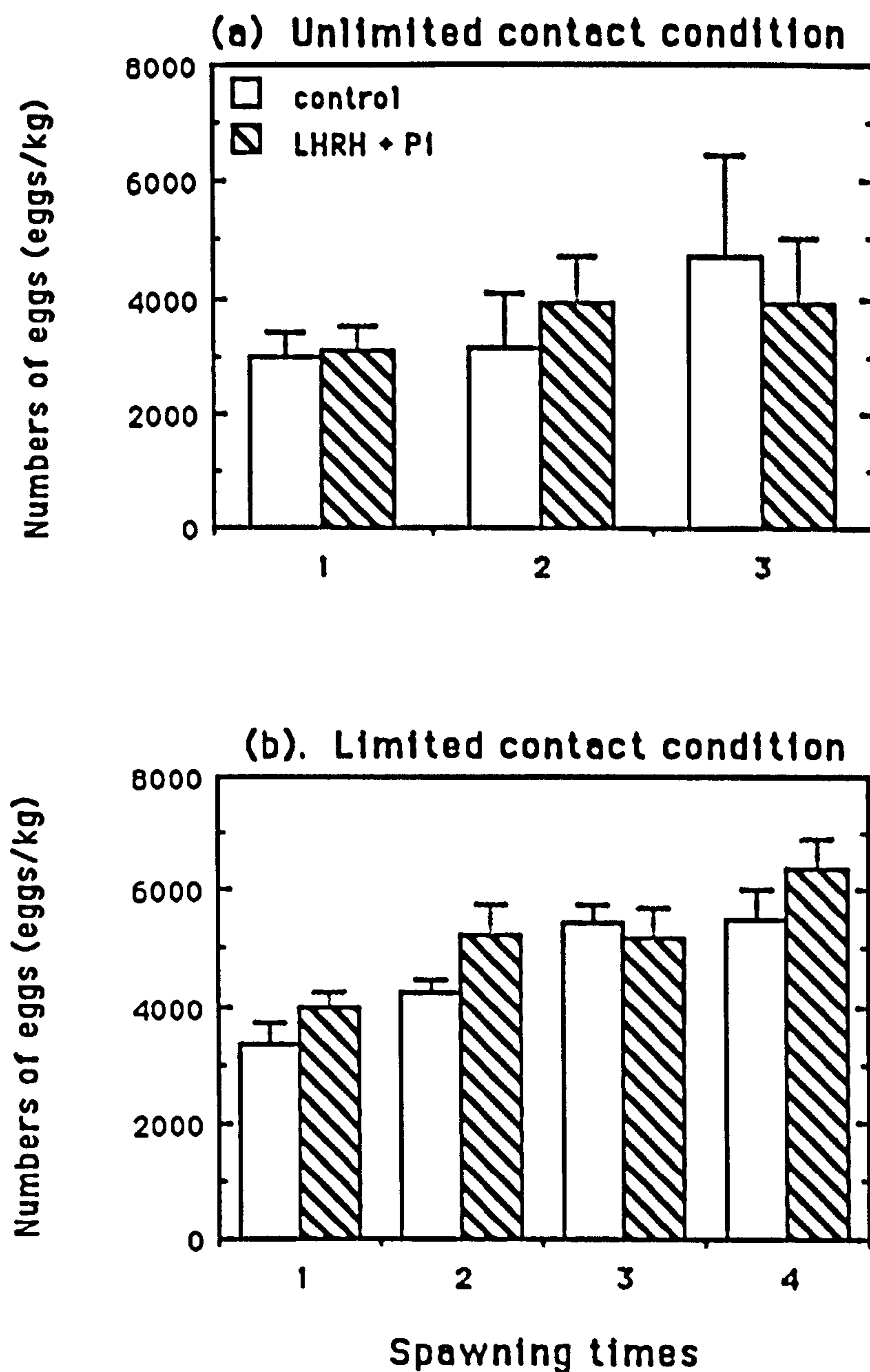


Figure 7.5: Effect of the  $100\mu\text{g/kg}$  body weight LHRH +  $0.1\text{ mg/kg}$  body weight pimozide on the relative fecundity (mean $\pm$ SE) of *O. niloticus* injected at 'day ten after spawning' (cycles 2, 3 and 4) compared with cycle 1 (the first spawning prior to hormone treatment). The fish were held under two different spawning conditions: (a) 'unlimited contact'; (b) 'limited contact'.



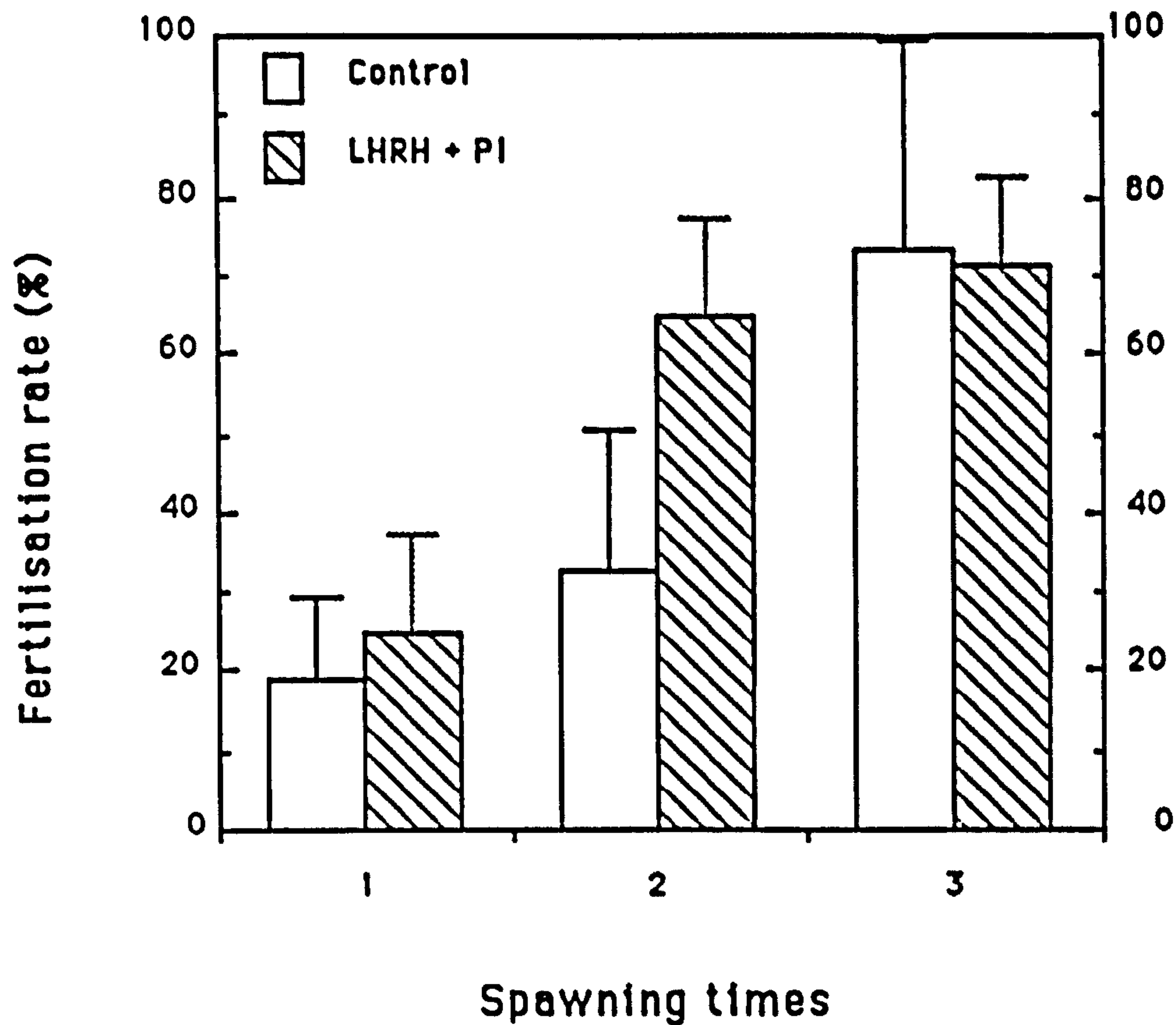


Figure 7.6: Effect of the 100 $\mu$ g/kg body weight LHRH + 0.1 mg/kg body weight pimozide on the natural fertilisation rates (mean $\pm$ SE) of *O. niloticus*. Fish were treated with 100 $\mu$ g/kg LHRH + 0.1 mg pimozide at 'day ten after spawning' and kept under the 'unlimited contact' condition for three spawnings.



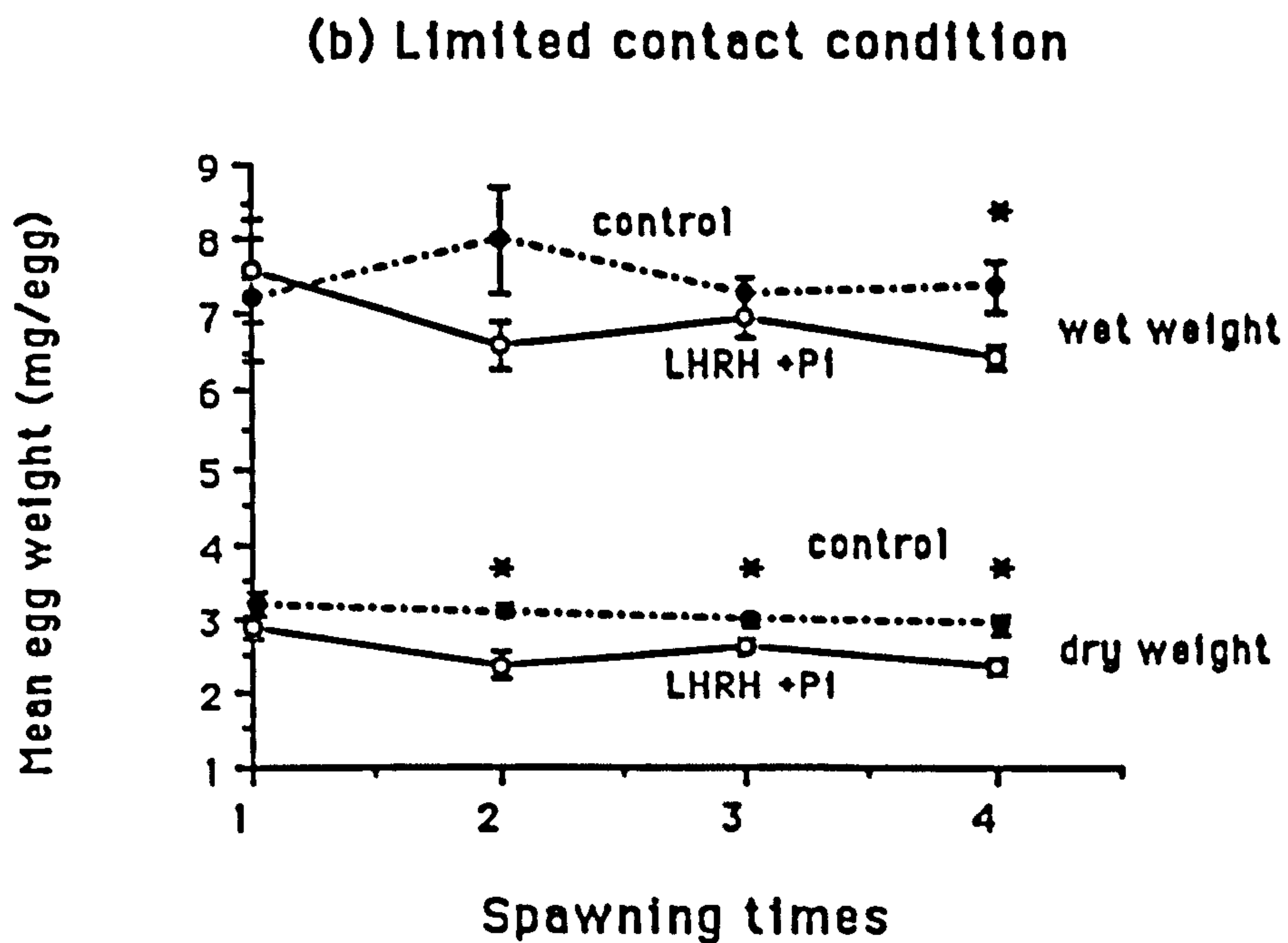
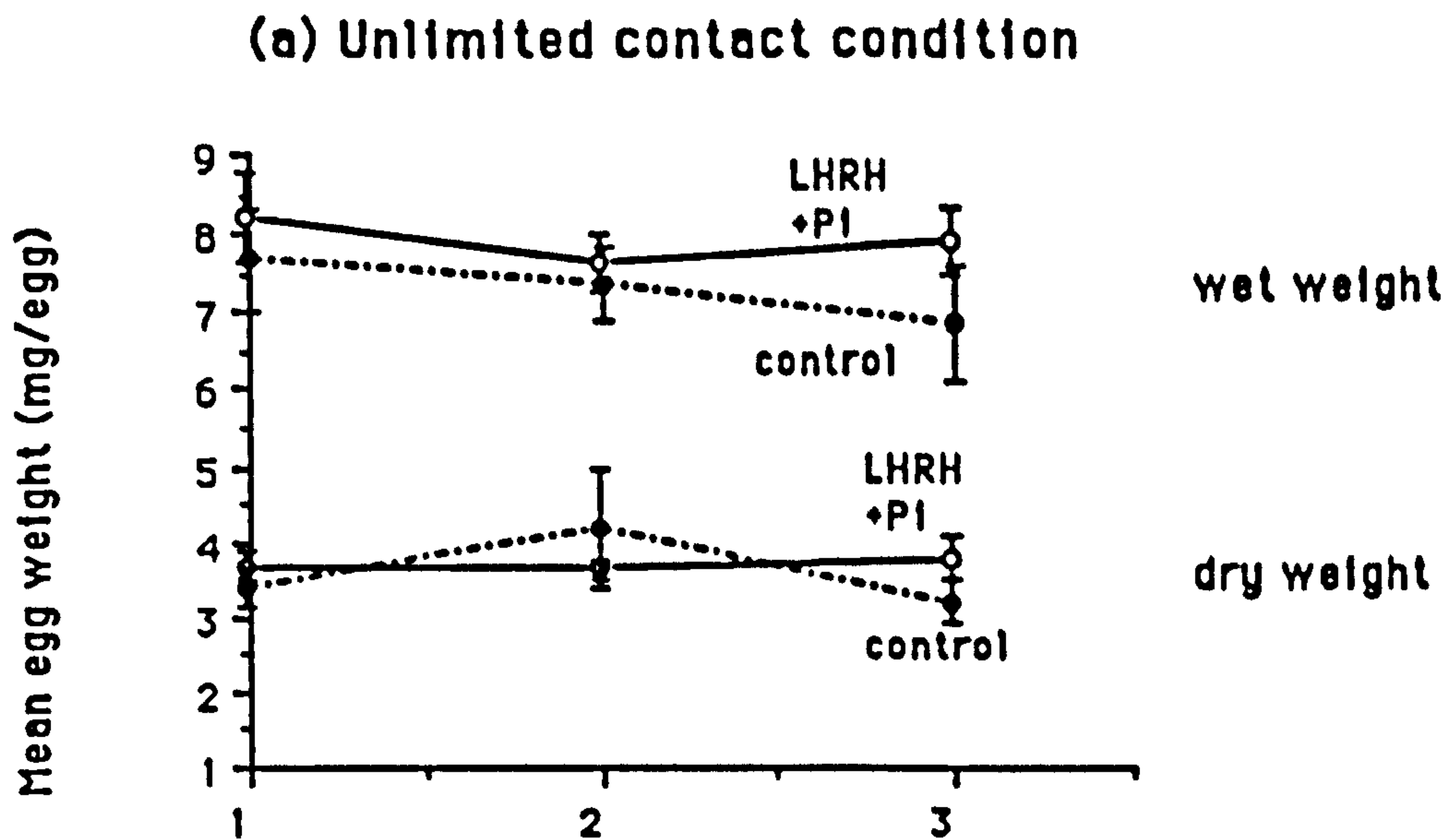


Figure 7.7 Effect of the 100 $\mu$ g/kg body weight LHRH + 0.1mg/kg body weight pimozone injected at 'day ten after spawning' on egg weight (mean $\pm$ SE) of *O. niloticus* prior to the treatments (cycle 1) and during the treatments (cycle 2, 3 and 4). The fish were held in different spawning conditions: (a) 'unlimited contact'; (b) 'limited contact'.



## 7.5 DISCUSSION

Luteinizing hormone-releasing hormones and their analogues are well known synthetic hormones for spawning induction in many fish species (Zohar, 1989; Peter, Lin, van der Kraak and Little, 1993). Although the ovaries of the fish selected in the present study, contained 55 to 65% of stage 6 oocytes prior to the hormone treatments (experiments 1, 2 and 3; Table 7.2), the hormone injections were ineffective in inducing the fish to spawn.

Handling stresses such as injections and bleeding during the experimental period should have had minimal affect on the fish spawning as demonstrated by a number of fish that spawned in both placebo and untreated controls. The various dosages of LHRH applied to the fish in experiment 1, 2 and 3 resulted in only marginal success i.e., the hormone treatments had lower or similar spawning percentage as the controls.

The initial ovarian stages of the selected fish prior to hormone treatment may be another factor which affected the outcome of the injection studies (experiments 1, 2 and 3). The fish selected by using external criteria showed a wide range in gonadal weight (Table 7.2), but there was no significant difference in the proportion of stage 6 oocytes in the ovaries of females between the three injection experiments. Of all the experiments, the ovaries of females

in experiment 3 had the highest GSI and also highest atretic oocytes. The GSI may therefore not be a reliable parameter for predicting maturation of *O. niloticus* females. This finding corresponds to de Vlaming et al., (1982) and West (1990) who had suggested the inaccuracy of GSI when compared with gonadal activity.

The rapid degradation of LHRH in fish may be a reason for the failure of these spawning induction trials. In goldfish for example, when a single injection of GnRH was administered, the hormone concentration in blood circulation peaked within 4 mins. By 12 mins, however, the hormone concentration was halved and then remained at low levels for 60 mins (Sherwood and Harvey, 1986). This rapid degradation and subsequent low levels of the GnRH may cause insufficient GnRH levels to induce further oocyte maturation and consequent ovulation in *O. niloticus* injected in experiment 1, 2 and 3 of the present study.

Results from the *in vitro* trials of Sherwood et al., (1988) showed that the fast releasing pellet (75% cholesterol + 25% cellulose) released more than 90% of hormone from the pellet within 24 hours, whereas in the slow releasing pellet (95% cholesterol + 5% cellulose), only 18 to 20% of the hormone was released from the pellet within 24 hours. The controlled release pellets were therefore designed to sustain hormone release in Experiment 4. All of these slow and fast releasing pellets used in the present study



(Experiment 4) contained a standard equivalent mean dose of 100 µg LHRH/kg fish body weight.

The *O. niloticus* females used in the hormonal implant studies appeared to be at the best gonadal stage for hormonal treatment. The ovaries of females selected for the implantation trial, contained the highest proportion of stage 6 oocytes and the lowest proportion of atretic oocytes when compared to the injection experiments (Table 7.2). Plasma samples of these fish also contained an average high T level (51.8 ng/ml) prior to the hormone treatments. This T level was similar to the mean T (60.2ng/ml) levels prior to spawning of the females in chapter 6. These may indicate that the fish selected for implantation may have been suitable for hormone treatment.

Unfortunately, neither the fast (75% cholesterol) nor slow (95% cholesterol) releasing pellets stimulated the fish to spawn despite the observation that the taGtH peaked at 6 hours post-implant. The plasma taGtH levels of both 75 and 95% cholesterol pellets increased from 17.4 ng/ml to 78.1 ng/ml and 75 ng/ml, respectively, within 6 hours post-implant. In contrast, the taGtH levels of the control fish only reached 15.3 ng/ml over the same period after implant (Figure 7.2). This suggests that the LHRH from the pellets was being released into blood stream of the fish.

In the present study, although the levels of taGtH peaked within 6 hours, unfortunately, these levels from the females given 75 and 95% cholesterol pellets dropped at day 1 post-implant and maintained similar low levels as the controls until day 45 post-implant, when the taGtH levels of one female given the 75% cholesterol pellet showed a dramatic increase and spawned. The taGtH levels in females treated with the 95% cholesterol pellet and the placebo controls remained at low levels until the end of the studies. This may indicate that the taGtH released in the first peak of both pellets might have subsequently inhibited the endocrine system of the fish. Therefore, over a period of 2 months, 37 and 0% of the females spawned in 75 and 95% cholesterol pellets, respectively (Table 7.5).

The taGtH profiles of the 75 and 95% cholesterol pellets were similar to the taGtH profile obtained from the injection of the same hormone combination in hybrid cichlid (Gissis et al., 1988), which peaked within 6 hours post-injection. Their results may confirm the too high LHRH dose used in the implantation experiment.

In seabass (Almendras, Duenas, Nacario, Sherwood and Crim, 1988) and milkfish (Marte, Sherwood, Crim and Harvey, 1987), optimal doses of mGnRH and sGnRH in cholesterol pellets were reported to be 16 to 40 µg/kg body weight. Whereas the low doses of 10 to 20 µg des-Gly<sup>10</sup> D-Ala<sup>6</sup>-LHRHa/kg body weight via silicone rubber implantation were



ineffective in inducing ovulation in brown trout (Billard, Reinaud, Hollebecq and Breton, 1984). In goldfish, the low (25 µg pellet) and high (125 µg pellet) doses of LHRHa (des-Gly<sup>10</sup>-[D-Ala<sup>6</sup>] had showed similar spawning results (Sokolowska et al., 1984). In contrast, Garcia (1989) studied different doses of LHRHa (des-Gly<sup>10</sup>-[D-Ala<sup>6</sup>] in seabass in relation to spawning and egg quality. His results showed that lower doses (37.5 and 75 µg/kg) induced all the fish to spawn with higher fertilisation and hatching rates than those from fish given the higher doses (150-300 µg/kg). In addition, higher doses of LHRHa in cholesterol implants was investigated in young male pink salmon, *Oncorhynchus gorbuscha* (9 mg/kg of sGnRH; MacKinnon and Donaldson, 1978) and female Atlantic salmon, *Salmo salar* (270 µg LHRHa/kg in cholesterol pellets and 6.7 mg [D-Nal(2)<sup>6</sup>]-LHRH/kg in silastic pellets; Crim, Glebe and Scott, 1986; Weil and Crim, 1983), all of these high dose hormones succeeded to accelerate precocious spawning of the juvenile fish.

Dosages of LHRH in the present study (experiment 1, 2, 3 and 4) were based on the effective dose of 100 µg/kg LHRHa reported by Gissis et al. (1988). All pellets used in this study contained 50 µg LHRH/pellet (100 µg/kg body weight). These doses may be too high for *O. niloticus* when compared with the doses of 16 to 75 µg/kg body weight in adult seabass (Almendras et al., 1988; Garcia, 1989) and in adult milkfish (Marte et al., 1987). In the present study, when

the high doses of LHRH pellets administered via 75 and 95% cholesterol pellets, both of the pellets resulted in only one taGtH peak at 6 hours post-implant and then the taGtH levels were suppressed. These low taGtH levels may reflect in the poor induced spawning success in these fish.

The precise timing for hormone administration is an important factor affecting successful spawning (Lam, 1982, 1985; Billard, 1989; Zohar, 1989). Ideally, hormonal inducement should coincide with high levels of T, GtH and  $17\alpha, 20\beta$ -P (Donaldson, 1986). In *O. niloticus* females, the ovaries at 'day ten after spawning' contained a maximum percentage of the volume fraction of stage 6 oocytes with the minimum proportion of atretic oocytes and was concomitant with the peak levels of  $Ca^{2+}$  and T as shown in chapter 6. This period may be the most suitable period for hormonal induction in *O. niloticus*. A trial (Experiment 5; section 7.3.2) was therefore carried out to investigate this precise period for hormone induction.

When the combination of 100  $\mu$ g/kg body weight LHRH + 0.1mg/kg body weight pimozide was injected at days 10 to 12 after spawning, only the hormone treated females under the 'limited contact' condition spawned earlier than the controls. In contrast, the hormone treated females in the 'unlimited contact' condition showed similar ( $P > 0.05$ ) spawning pattern as the control (Figure 7.3; Table 7.6). This result corresponds to Gissis et al (1991) who



succeeded inducing 9 fish from 11 fish to spawn with a single injection of 10 µg/kg LHRHa + 5 mg domperidone into the individually housed hybrid cichlid at day ten after spawning. Thus, the ovarian stages of *O. niloticus* plays an important role for using exogenous hormone to induce synchronous spawning in this fish.

The environmental spawning conditions especially the social interaction between males and females are additional factors, which affect natural spawning. Lowe-McConnell (1959) suggests that visual stimulation may be an important factor throughout the life cycle of cichlid fish. For example in *O. mossambicus* which were maintained in complete isolation from social stimulation, the first spawning of the females was delayed by about 10 days when compared to that of socially stimulated fish (Silverman, 1978a). In similar studies with adult *O. mossambicus*, which were held under unlimited social contact condition, the reproductive activity of the fish was enhanced when compared to those of isolated females (Silverman, 1978b).

In addition, Chieng (1973) reported that the spawning rates of 'angelfish', *Pterophyllum scalare*, which were exposed to visual and chemical stimulation ('social with limited contact' in the present study), were better than those subjected either to only visual, chemical or control ('social unlimited contact' in the present study) conditions. All of these females spawned better than those

females kept in the completely isolated condition. His study suggested that spawning success of this species may be affected by their spawning conditions.

In the present study, when hormonal therapy was applied under the 'limited contact' condition (only visual no physical contact was allowed), the fish showed a clear positive response to the LHRH + pimozide injection. Whereas in the 'unlimited contact' fish (visual and physical contact were allowed) showed a negative response to the hormone injection. The condition of ovary and spawning environment of the females prior to and during hormone treatment were, therefore, identified as the most important factors for successful hormone induction of *O. niloticus* and may explain the negative responses observed in the hormonal injection and implantation experiments.



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## CHAPTER 8

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## 8. GENERAL DISCUSSION

Unwanted spawning of tilapias results in rapid overcrowding of fish in grow out systems. Consequently the harvest often contains a large size range of fish from which only a small proportion may be of the required size (250 - 300g).

Numerous attempts have been made to minimise and control the spawning of tilapias under cultural condition. The use of polyculture with predators, high density culture and monosex male culture (Mires, 1977; Sin and Chiu, 1983; Verani et al., 1983 and Hanson et al., 1983) have all achieved some measure of success. Monosex culture of males which is produced by sex inversion is the most popular method for culture (Mires, 1977; Guerrero, 1982; Hanson et al., 1983; section 5.3.2). In this technique, sex of tilapia fry is reversed by feeding an exogenous male steroid to the sexually undifferentiated fry to obtain high percentage of males. The efficacy of hormonal sex reversal depends on type and dosage of male hormone used, methods and duration of treatment, species, age and size of fish prior to treatment (Mires, 1977; 1983). Yamamoto (1969) reported that the hormone sex reversal treatment should be started before the onset of sexual differentiation.

Timing of the onset of sexual differentiation published in literature is very variable and often different criteria are used. If the age at sexual differentiation is



considered then *O. niloticus* fry should be treated before they are 30 days old (Alvencia-Casauay and Carino, 1988). Guerrero (1977; 1982) suggested the fry which have just completed yolk absorption are most suitable. Tayamen and Shelton (1978); Nakamura and Iwahashi (1982) and Hopkins et al., (1979) on the other hand used the fry length of shorter than 12mm as the critical size for hormonal sex reversal of tilapias. The criterion of fry size (less than 12mm), may be unreliable since the size of the fry depends on several cultural conditions such as stocking density, food, etc. Therefore, a population of similar size (e.g., less than 12mm) may consist of mixed age classes in which the older but smaller fry may have already completed their sexual differentiation.

In addition, the present study suggests that the stocking densities of fry may affect the onset of sexual differentiation. In this study, 5 day old *O. niloticus* fry were stocked at an initial densities of 2 (low), 10 (medium) and 20 fry/l (high). At these densities, 30 and 45% of the fry stocked at the medium and high densities were sexually differentiated by 11 day post-hatch. The mean total length of these fry were 11.9 and 11.2 mm, respectively. The average total length of these fry, however, was shorter than the 12 mm recommended for use in sex reversal treatment. Fry of the same age which were initially stocked at 2 fry/l had a total length of 12.3 mm and showed sexually undifferentiated gonads. Evidence from

the present study indicated that fry stocked at higher densities had sexual differentiated gonad earlier than those stocked at lower density.

Commercial all male seed production requires large numbers of fry of the same age or size. The mass production of tilapia fry is constrained by their reproductive biology. At present, tilapia seed are produced mainly from natural spawning in pond or hapas. Unlike salmonids and carps, tilapias are asynchronous spawners and have a low fecundity (700 - 2,000 egg/spawn) consequently large numbers of broodstock need to be managed. For example, the production of 100 tons of tilapia (250g) requires an estimated 1,000,000 fry/year and requires at least 100 spawnings / month (Rana, 1986).

Fecundity or egg numbers/spawn can be estimated by either directly counting the ovoposited eggs or estimated from mature oocyte numbers digested in Gilson's fluid. Recently, stereological techniques using numerical density equation (section 4.1.2) have been successfully used to estimate egg numbers in some marine species (Emerson et al., 1990). Similar stereological estimation used in the present study gave results comparable to those from the Gilson's fluid method (section 4.4.6; Emerson et al., 1990). The Gilson's fluid technique for fecundity estimation, however, has several drawbacks (Emerson et al., 1990). In the present study the classification of oocyte stages in Gilson's fluid



were based on 99% confidence limits of the absolute diameters, which were determined from histological slides of ovaries of the oocyte stages. In this technique, there was considerably overlap between oocyte stages based on their diameters (Figure 4.7). This overlap depended on the maturity of the gonads. When gonads with low and high GSIs were pooled the overlap of stages 5 and 6 oocytes was 37%. The separation of data according to gonad size reduced this overlap to 24%. The extent of the overlap of this technique was confirmed by histological evidence. For example, when the stage 6 oocytes which were classified solely on their diameter size were examined histologically, they contained 7.5 - 60% and 1 - 90% of stages 4 and 5 oocytes, respectively (Table 4.7).

The structure of *O. niloticus* oocytes in different stages is shown in chapter 3 of this present study. When oocytes were highlighted with polychrome stain, six different oocyte stages including stage 2, 3, 4, 5, 6 and atretic oocytes were easily identified (Table 3.1) from a single slide. Atretic oocytes were excluded from all previous classification of tilapia oogenesis (Dadzie, 1970; Latif and Saad, 1973b; Babiker and Ibrahim, 1979a). With this staining method, atretic oocytes were easily detected by the changing yolk affinity from the basophilia into acidophilia. These changes were commonly observed in the spent female ovaries (Table 3.1).

Determination of ovarian volume fractions by the intersection method was the simplest and quickest among all the stereological techniques. Due to the use of a multipurpose graticule, this intersection technique required only 2.6 mins for investigating a sample (section 4.4.3; Table 4.4). The oocyte classification of this method, in addition, was based on the oocyte structures, which was a more accurate method than using GSI to determine fish maturity. For example, the structure of the pre-treated LHRH ovaries (chapter 7; Table 7.3), showed that the highest GSI (2.7%) of the females contained a lower proportion (56%) of stage 6 and a higher proportion (25%) of atretic oocytes than lower GSIs. In the females with lower GSIs (1.9 - 2.6%), the ovaries contained a higher proportion (59 - 71%) of stage 6 and a lower proportion (5-11%) of atretic oocytes. This evidence highlights the potential inaccuracy of GSI in determining the maturity of *O. niloticus* broodstock. Therefore, the volume fraction of stage 6 oocytes may be the most accurate and appropriate method for determining maturity in tilapias.

Nevertheless, GSI is a widely used indicator to determine the state of maturity in many teleost species. In the present study (section 5.4.2), low GSIs in the range of 0.3 - 0.5% were found in young *O. niloticus* females between 14 to 18 weeks. The mean GSIs increased rapidly to 2 - 3.6% in maturing and mature females between 20 to 24 weeks. In



addition, the mean ovarian volume fraction of stage 6 oocytes was significantly correlated with GSIs ( $r^2 = 0.84$ ;  $P < 0.05$ ; Figure 4.4). For example, the GSIs of immature females averaged 0.3%, and stage 6 oocytes volume fraction occupied only 6.1 - 12.1% in the ovary. When the mean GSIs from mature females increased and ranged between 3 - 3.9%, 70 - 82% of the ovaries were occupied by stage 6 oocytes (Table 4.1, 4.3c). By 22 weeks the mean volume fractions of stage 6 and atretic oocytes rose rapidly to 72 and 4.6%, respectively. While at 24 weeks average stage 6 oocytes declined to 68% and atretic oocytes volume fractions increased to 6%. From the above results, the present study concluded that the *O. niloticus* females may attain maturity by 22 weeks.

The maximum volume fraction of stage 6 oocytes in 22 week old females corresponded to peak levels of  $Ca^{2+}$ , T and  $E_2$ . By 24 weeks only the  $E_2$  levels declined whereas the other levels remained high. These results confirm that the *O. niloticus* females complete vitellogenesis and attain maturity at 22 weeks. At this time, the average female size was 14.1cm (57.3g). This study, however, was specially designed to follow puberty and therefore, the spawning ability of the stocks was not investigated. It may be possible that if the females were maintained under ideal spawning conditions they may have commenced spawning at a different age and size.

Previous studies on tilapia reproductive biology have been carried out on fish of unknown age and spawning history. This has confounded our understanding of tilapia reproductive cycles. The present study was therefore carried out with fish of known age and spawning history and aimed to control and synchronize the spawning of tilapias.

Females that were allowed to spawn naturally and then robbed of their eggs (non-mouthbrooding females), were able to enter a new reproductive cycle earlier and it is, therefore, possible to increase the numbers of spawnings per season (Fishelson, 1966). Rana (1986) also reported that the shorter spawning cycles of non-mouthbrooding were not universal and were found only in the females subjected to a low stress environment. The interspawning intervals can vary between females and between spawnings of the same female. For example, Smith and Haley, (1987) reported 25 and 45 day intervals between spawning cycles of non-mouthbrooding and mouthbrooding females, respectively. Whereas Siraj et al., (1988) reported shorter interspawning intervals of 7 to 10 days for non-mouthbrooding *O. niloticus* in hapas. In the present study, the ovarian recrudescence of the non-mouthbrooding *O. niloticus* females was investigated (section 6.4.1). When the spawning history of the females was fixed after their first detected spawning, the females spawned at the median spawning interval of 13 days (11 to 29 days).



Histological studies of ovaries sacrificed at days 1, 5 and 10 after the first, second or third spawning showed that atretic oocytes were resorbed and immature oocytes developed rapidly into mature stage 6 oocytes within a period of 5 - 10 days (section 6.4.1). The *O. niloticus* ovaries occupied by stage 6 oocytes in this study peaked at 65 to 72% at day 10 after spawning; indicating that the ovarian recrudescence of non-mouthbrooding *O. niloticus* females required a short period of 10 days after each spawning and the consequently spawning could occur approximately 3 days later.

Moreover, the levels of oestradiol-17 $\beta$  ( $E_2$ ) increased from day 1 after spawning, peaked at day 5 and reduced at day 10 after spawning. These  $E_2$  levels confirmed that the oocytes at day 5 after spawning were in advanced vitellogenesis and vitellogenesis was completed by day 10 after spawning. Although this trend occurred between a period of 13 and 28 days, this pattern was similar to the trend of  $E_2$  in annual spawning teleosts, such as, winter flounder, *P. americanus*, grey mullet, *M. cephalus*, rainbow trout, *O. mykiss* and goldfish, *C. auratus* (Campbell et al., 1976; Whitehead et al., 1978; 1983; Crim and Idler, 1978; Azoury and Eckstein, 1980; Scott et al., 1983; Kobayashi et al., 1986; 1988; 1989). The profile of  $E_2$  for *O. niloticus* in the present study, however, was different to that reported for non-mouthbrooding *O. mossambicus* by Smith and Haley (1987).

These authors observed a bimodal  $E_2$  peak at 10 and 25 days post-spawning.

The levels of T may also be used as an indicator for spawning (Fitzpatrick et al, 1987). In the present study, the trends of T and  $Ca^{2+}$  during ovarian recrudescence were similar and had peaked by day ten after spawning. The timing of the maximal levels of T and  $Ca^{2+}$  coincided with that of stage 6 oocytes. This trend also coincided with the presence of a key ovarian enzyme (3 $\beta$ -hydroxysteroid dehydrogenase; 3 $\beta$ -HSD) involved in steroid biosynthesis at 10 day after spawning (Smith and Haley, 1987). All these indicators may confirm that the ovary at day 10 after spawning is fully mature and oocytes may be close to ovulation. The recognition of this period may be important for successful hormonal administration (section 7.4.1). When females were categorized by their external characteristics prior to LHRH administration, all attempts at induced spawning were unsuccessful.

The duration of the spawning cycle in females may depend on the absolute peak levels of T and  $E_2$  during the ovarian recrudescence of that cycle. When the average high levels of both T and  $E_2$  (60.4ng/ml and 38.4ng/ml, respectively) were found, 87% of the females spawned at a median time of 13 days (9 - 45 days) whereas females which had lower average peak levels of both T and  $E_2$  (34.6ng/ml and 28.6ng/ml, respectively), exhibited a long spawning cycle



with a median time of 28 days (9 - 44 days). These peak levels of T and E<sub>2</sub>, however, showed no correlation to the interspawning intervals ( $r^2 = 0$ ). During these long spawning cycles, several low peak levels of T, E, and Ca<sup>2+</sup> were detected every 9 to 14 days in unspawned females (section 6.4.2). These periods coincided with the recrudescence period of 10 days and the median of short spawning cycles (13 days; section 6.4.1). The results suggest that additional triggers may be implicated in final ovulation and spawning.

Although ovaries had completed exogenous vitellogenesis within 10 days after spawning, absolute levels of T in some females may not have been sufficient enough for ovulation and thus no spawning occurred (Santos et al, 1986). These low T levels were also reported to delay the spawning period in coho salmon, *O. kisutch* (Fitzpatrick et al., 1987). In unovulated mature female common carp, *C. carpio* also showed very low peaks of T and GtH (Santos et al., 1986). It is possible that the females in the present study which did not spawn may have had insufficient T and/or GtH levels. This requires further investigation.

The low spawning frequency which was reported by Mires (1982) may be due to the conditions used for spawning tilapia. In his study, females were held in isolated aquaria and introduced to a male aquarium when they showed evidence of protruding genital papillae. On many occasions,

however, the females failed to spawn and this was attributed to aggressive behaviour by the male. This failure may be due to suboptimal levels of T of the females (section 6.4.2 and Santos, 1986; Fitzpatrick et al., 1987 and Kobayashi, et al, 1989).

In order to improve the predictability of tilapia spawning in the present study, slow (95% cholesterol) and fast release (75% cholesterol) pellets containing approximately 50  $\mu\text{g}$  LHRH/pellet (average 100  $\mu\text{g}/\text{kg}$  body weight) were administered to *O. niloticus* females. Although the ovaries of these selected females contained high (65%) proportions of stage 6 oocytes and high testosterone levels prior to the implantation, these hormone pellets proved ineffective in inducing the fish to spawn.

The taGtH of the females given both the 75 and 95% cholesterol pellets reached a peak within 6 hours whereas the controls stayed at low levels. This may suggest that the hormone was released from the pellets and was successful in stimulating the production of taGtH. The levels of taGtH, however, were only sustained for 6 hours post-implant. This taGtH trend in the present study were similar to the taGtH profile obtained by injection of 100 $\mu\text{g}$  GnRHa in hybrid tilapia (Gissis et al., 1988; 1991). Although the average dose of LHRH in the pellets was similar to their studies (approximately 100 $\mu\text{g}$  LHRH/kg female), this dose may have been too high for the *O.*



*niloticus* females via implantation. This dose was also higher than those (16 - 74  $\mu\text{g/kg}$ ) used for seabass and milkfish (Marte et al., 1987; Almendras et al., 1988; Garcia, 1989). In addition, Garcia (1989) showed that lower doses of 37.5 and 75  $\mu\text{g/kg}$  in cholesterol pellets induced seabass to spawn and the eggs had higher fertilization and hatching rates than those given with higher doses (150 to 300  $\mu\text{g/kg}$ ). Their results suggest that the doses of LHRH contained in the pellets used in section 7.4.1 may be too high. This high dose may have depressed the GnRH production after 6 hours post-implant and may result in no spawning improvement of the females implanted with 75 and 95% cholesterol pellets. The dose response to LHRH implantation needs further investigation.

Another factor that may be implicated in successful induced spawning may be the subtle differences in ovarian development, spawning history between females and their spawning conditions. To overcome this, a trial (section 7.4.2) was conducted by injecting the females kept in isolation and in normal communal spawning conditions at 10 days after spawning (section 6.4.1) with 100 $\mu\text{g}$  LHRH + 0.1 mg pimozide/kg body weight. The females which were held in isolation, spawned within 2 - 6 days of the inducement. In contrast, the hormone treated fish held in the communal spawning tanks in which the fish had unlimited social contact, showed similar spawning cycles to that of the controls. These fish did not respond to the hormone

treatment although the ovaries of these fish were sensitive to LHRH. Therefore, the degree of contact between males and females may be one of the most important factors for successful spawning of tilapias. If the peak levels of T in females are too low (e.g., less than 30 $\mu$ g/ml), it is possible that the males may successfully attack or kill the females instead of stimulating the females to spawn (Fishelson, 1966; Rothbard, 1979; Mires, 1982). When the females were protected by plastic partitions, the males were able to stimulate the females via visual and chemical (pheromone) contacts until they were ready to spawn (section 7.4.2). This observation corresponds to Chien (1973) who reported that in angelfish, *Pterophyllum scalare*, exposed only to visual and chemical stimulation (social with limited contact in the present study) had a higher spawning frequency than those maintained under either only visual, chemical or control (control = social with 'unlimited contact' in the present study) condition.

Based on the results of the present study, it could be concluded that the timing of hormone inducement and spawning conditions affect the successful spawning of the *O. niloticus* females. The reproducibility of these results will need to be confirmed on larger numbers of females.

The spawning history and spawning conditions of females may affect the quality and quantity of tilapia eggs. In the present study, the egg numbers of non-mouthbrooding females



(section 6.4.1) depended on the spawning history of the females. Individual females which had spawned for the second or the third time ovulated more eggs than those which had spawned for the first time. Similar results were also reported for other tilapia species (Peter, 1983). When the females were pooled, their fecundity between different spawning times were, however, not statistically different. In addition, egg quality which was measured by fertilisation rates, could be related to the peak levels of  $E_2$  in each spawning cycle. High fertilisation rates (86 - 96%) corresponded to high levels of  $E_2$  (48.3ng/ml), whereas lower  $E_2$  levels (17.6 - 32.5ng/ml) resulted in 0 - 74.4% fertilisation rates (section 6.4.2). These fertilisation rates in the present study, however, showed no statistical differences between different spawning times.

In addition, the size of *O. niloticus* eggs, measured by egg weight, may be affected by period of spawning cycles. For example, the females which were successfully induced to spawn every 10 - 12 days after each spawning time, produced significantly smaller eggs than those females spawned in the longer spawning cycles (Table 7.8; Figure 7.7).

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## CHAPTER 9

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## 9. CONCLUSIONS

1. By 11 days post-hatching, 30 and 45% the fry which were initially stocked at medium (10/l) and high (20/l) densities, respectively, had sexually differentiated gonad when compared to those from lower density (2/l) which showed unsexually differentiated gonad.
2. *O. niloticus* oocytes were classified into six stages: stages 2, 3, 4, 5, 6 and atretic oocytes (Table 3.1). The atretic oocytes were characterised by changes of yolk affinity from basophilia into acidophilia using polychrome stain.
3. Stereological techniques using numerical density equation estimated similar oocyte numbers to those derived from Gilson's fluid method.
4. The Gilson's fluid method which used 99% confidence limits of actual oocyte diameters for staging oocyte in the present studies, evidently showed considerable overlap (up to 37%) between different oocyte stages. The degree of overlap was reduced to 24% when GSI levels was considered.

5. The extended overlap of the Gilson's fluid technique was studied by histological evidence. The stage 6 oocyte separated by this method were examined histologically, they contained 7.5 - 60% and 1 - 90% of stages 4 and 5 oocyte, respectively.
6. Volume fractions (%) of different oocyte stages were classified based on their histological structures. Thus, this method should be more accurate in staging oocyte than that of Gilson's fluid and GSI.
7. Ovaries with high GSI may contain high volume fractions (%) of atretic and low stage 6 oocyte.
8. The volume fractions of different oocyte stages estimated from graphic (graph paper), mass (plastic sheet weighing) and intersection methods (multipurpose graticule) yielded similar estimations. The intersection method required only the short period of 2.6 mins/sample whereas the others needed 12.1 and 11.1 mins/sample, respectively.
9. The volume fraction of stage 6 oocyte was significantly correlated with GSIs ( $r^2 = 0.84$ ;  $P < 0.05$ ), therefore, it could be used as an index to determine maturity of *O. niloticus* females.



10. Ovaries of *O. niloticus* females at 14 to 18 week old (GSI = 0.3%), contained 6.1 - 12.1% stage 6 oocyte volume fractions. By 22 weeks these mean volume fractions peaked to 72%, meanwhile atretic oocyte were also detected (4.6%). Average stage 6 oocyte at 24 weeks declined to 68% and the atresia increased to 6% of ovary. The trend of stage 6 oocyte was similar to  $E_2$  levels which also peaked by 22 weeks prior to subsequently reduced at 24 weeks. The females showed significantly high  $Ca^{2+}$  and T levels by 20-24 weeks. Therefore, the *O. niloticus* females (14.1cm; 57.3g) attained maturity at the age of 22 weeks.
11. Although male GSIs showed no significant differences throughout the present study, the males showed secondary sexual characteristics and their testes contained spermatozoa by 16 weeks . Significant increase in total calcium ( $Ca^{2+}$ ), testosterone (T) levels were found at 22 weeks. The *O. niloticus* males (14.7cm; 70.2g), therefore, attained maturity by 22 weeks.
12. The weight of the males was significantly ( $P<0.05$ ) higher than those females by 18 weeks.

13. During ovarian recrudescence of non-mouthbrooding *O. niloticus* females, GSI, stage 6 oocyte volume fractions,  $\text{Ca}^{2+}$  and T peaked by 10 days after spawning. While  $\text{E}_2$  peaked by 5 days and reduced by 10 days post-spawning. These suggested that the ovaries at day 5 after spawning were in vitellogenesis which was completed by day 10 after spawning.
14. Levels of  $\text{E}_2$  during spawning cycles were significantly correlated to the volume fractions of stage 6 oocyte ( $r^2 = 0.79$ ;  $P < 0.05$ ).
15. Ultrastructural studies of oocyte at different periods after spawning, showed changes of thecal, granulosa, microvilli, chorion and microfilament at days 1, 5 and 10 after spawning.
16. Spawning cycles of the non-mouthbrooding *O. niloticus* females were grouped into cohorts of short (9-15 days) and long (22-45 days) spawning cycles which occurred at the median time of 13 and 28 days, respectively. The overall spawning cycle, however, was found to be 15 days.
17. Only one female (No. 834) from eight non-mouthbrooders spawned at 9 - 15 day intervals and this co-incided with every peak values of  $\text{Ca}^{2+}$ , T and  $\text{E}_2$ . The other 7 females spawned after those several minor peaks had lapsed.



18. Peak levels of T and E<sub>2</sub> may affect the period of the spawning cycles. When both of the mean levels were high, 87% females spawned in the short spawning cycles. In contrast, when both of the levels were low, 63% of the females spawned in the long spawning cycles and the other spawned in the short cycles.
19. The peak levels of E<sub>2</sub> during each spawning cycle was significantly correlated ( $r^2 = 0.49$ ;  $p < 0.05$ ) with fertilisation rates of eggs. The high peak levels of E<sub>2</sub> in females resulted in high fertilisation rates.
20. Spawning history affected the number of eggs oviposited. The non-mouthbrooding *O. niloticus* females which had spawned previously tended to release more eggs than those had just spawned for the first time.
21. The eggs numbers and fertilisation rates of different spawning times were not statistical differences.
22. The females which were successfully induced to spawn every 10-12 days using 100 µg LHRH/kg + 0.1 mg pimozone/kg body weight, produced significantly smaller egg sizes than those females spawned in the longer spawning cycles.

23. Administration of D-Ala<sup>6</sup>-Gly<sup>10</sup>-LHRH either by injection (10-300 µg/kg LHRH + 0.1mg/kg pimozide) or cholesterol pellet implantation (100 µg/kg LHRH) had no effect on the efficacy of induced spawning when the females were selected by external characteristics.
24. Spawning conditions and timing at "10 days after spawning" for hormonal administration were found to affect the spawning success of the non-mouthbrooding *O. niloticus* females.
25. Females which were held in isolation and allowed only visual but no physical contact with males were successfully induced to spawn by 2 to 6 days post-injection of the 100 µg/kg LHRH + 0.1mg/kg pimozide administered at 10-12 days after each spawning.
26. Under communal spawning condition where the fish had unlimited social contact between males and females, the females did not respond to the hormone treatment although their ovaries were sensitive to LHRH. Therefore, the degree of contact between males and females may be one of the most important factors for successful spawning of tilapias.



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## APPENDICES

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## APPENDIX 1

### 1. TECHNIQUES USED FOR HISTOLOGICAL PREPARATION OF SLIDES

#### 1.1 FIXATIVE "Bouin's solution"

|                               |    |    |
|-------------------------------|----|----|
| 40% Formaldehyde              | 25 | ml |
| Saturated aqueous picric acid | 75 | ml |
| Glacial acetic acid           | 5  | ml |

#### 1.2 TISSUE PROCESSING FOR HISTOLOGICAL STUDY

##### 1.2.1 Times (hours) for paraffin wax embedding

|                        |   |
|------------------------|---|
| 80% Methylated spirit  | 1 |
| 100% Methylated spirit | 2 |
| 100% Methylated spirit | 2 |
| 100% Methylated spirit | 2 |
| 100% Alcohol           | 2 |
| 100% Alcohol           | 2 |
| Chloroform             | 2 |
| Chloroform             | 1 |
| Molten wax             | 1 |
| Molten wax             | 2 |
| Molten wax             | 2 |

##### 1.2.2 Times (days) for tissue processing for historesin embedding

|   |      |
|---|------|
| 80% Ethanol                               | 1    |
| 90% Ethanol                               | 1    |
| 95% Ethanol                               | 1    |
| 50:50 (Infiltration solution:95% ethanol) | 3-7  |
| 75:25 (Infiltration solution:95% ethanol) | 3-7  |
| 100% Infiltration solution                | 7-14 |



### 1.3 PROCEDURES FOR HISTOLOGICAL STAINING

#### 1.3.1 Haematoxylin and Eosin Stain

| <u>Chemicals</u>         | <u>Time (mins)</u> |
|--------------------------|--------------------|
| Xylene                   | 5.0                |
| Alcohol I                | 2.0                |
| Methylated spirits       | 1.5                |
| Running water            | 1.0                |
| Haematoxylin             | 5.0                |
| Running water            | 1.0                |
| Acid alcohol             | 1.0                |
| Running water            | 1.0                |
| Scott's tap water        | 1.0                |
| Examine under microscope |                    |
| Running water            | 1.0                |
| Eosin                    | 2-5                |
| Quick wash in tap water  |                    |
| Alcohol II               | 1.5                |
| Alcohol I                | 2.0                |
| Xylene                   | 5.0                |

After xylene, the slides were mounted with D.P.X and covered with cover slips. The slides were then allowed to dry at room temperature before examination.

### 1.3.2 Heidenhain's iron haematoxylin

#### Preparation:

(a) The alum bath for a mordant:

|   |        |
|---|--------|
| $[(\text{NH}_4)_2.\text{Fe}_2(\text{SO}_4)_4.24\text{H}_2\text{O}]$ | 5 g    |
| Distilled water   | 100 ml |

(b) The alum bath for a differentiator:

|   |        |
|---|--------|
| $[(\text{NH}_4)_2.\text{Fe}_2(\text{SO}_4)_4.24\text{H}_2\text{O}]$ | 2.5 g  |
| Distilled water   | 100 ml |

(c) The haematoxylin bath:

|                  |         |
|------------------|---------|
| Haematoxylin     | 0.5 g   |
| absolute alcohol | 10.0 ml |
| Distilled water  | 90.0 ml |

#### Procedures:

|                |            |
|----------------|------------|
| Mordant        | 1.50 hours |
| Rinse in water | 5 mins     |
| Haematoxylin   | 1.50 hours |
| Rinse in water | 5 mins     |
| Differentiator | 5-10 mins  |

During differentiated periods, the differentiation process was controlled by taking the slide out of the differentiator, dipping into water and then examining under a microscope. Repeat the process until the sections were destined.

|                                      |        |
|--------------------------------------|--------|
| Rinse in water                       | 5 mins |
| Dehydrate in a series of alcohol     |        |
| Clear in xylene and mount in balsam. |        |



### 1.3.3 Polychrome staining

| <u>Chemicals</u>       | <u>Time (mins)</u> |
|------------------------|--------------------|
| 1% periodic acid       | 10                 |
| Running water          | 5                  |
| Schiff's reagent       | 30                 |
| Running water          | 5                  |
| Bromophenol blue       | 25                 |
| 0.5% Acetic acid       | 20                 |
| Running water          | 3                  |
| Ehrlich's haematoxylin | 40                 |
| Buffer pH 7.4-8.0      | 2 washes           |

## APPENDIX 2

### 2: TECHNIQUES USED FOR ELECTRON MICROSCOPICAL STUDY

#### 2.1 TISSUE PROCESSING FOR EM EMBEDDING

|  |    |      |
|--|----|------|
| 90% Ethanol                            | 30 | mins |
| 100% Ethanol                           | 30 | mins |
| 100% Ethanol                           | 30 | mins |
| 50:50 (Ethanol:epoxy-propane)          | 30 | mins |
| 25:75 (Ethanol:epoxy-propane)          | 30 | mins |
| 100% Epoxy-propane                     | 30 | mins |
| 50:50 (Epoxy-propane:embedding medium) | 2  | h    |
| 25:75 (Epoxy-propane:embedding medium) | 2  | h    |
| 100% Embedding medium                  | 1  | h    |
| 100% Embedding medium                  | 1  | h    |

#### 2.2 REYNOLD'S LEAD CITRATE STAINING FOR EM

##### Reynold's lead citrate

|                |        |
|----------------|--------|
| Lead nitrate   | 1.32 g |
| Sodium citrate | 1.76 g |
| NaOH           | 8.0 ml |

Made up with distilled water to 50 ml solution.



## APPENDIX 3

### 3. HORMONAL ASSAYS PREPARATION

#### 3.1 CALCIUM DILUTING SOLUTION

|                                |    |    |
|--------------------------------|----|----|
| Nitric acid (spectosol)        | 10 | ml |
| Lanthanum chloride (spectosol) | 10 | g  |

Make up with deionised water to a final volume of 1000 ml.

#### 3.2 STEROID PHOSPHATE BUFFER

|                             |        |
|-----------------------------|--------|
| Disodium hydrogen phosphate | 8.88 g |
| Sodium dihydrogen phosphate | 5.82 g |
| Sodium chloride             | 4.50 g |
| Gelatine                    | 0.50 g |

Make up to 500 ml solution with Nano Pure water.

### 3.3 PROCEDURES FOR SET UP ATOMIC ABSORPTION SPECTROPHOTOMETER

1. Check and drain vessel if necessary.
2. Fit in calcium bulb.
3. Turn GAIN and LAMP1 to zero.
4. Switch on power and set BGKD to AA, SLIT to 0.7, WAVELENGTH to 423 nm, SIGNAL to LAMP1 and MODE to CONT.
5. Adjust lamp/energy to 20 with LAMP1 and leave for 15 to 30 minutes.
6. Set SIGNAL to ABS
7. Maximize lamp/energy reading with: a)GAIN, b)WAVELENGTH, c)moving the bulb within the bulbmounting and d)the two position knobs.
8. Switch on air compressor, turn on the main acetylene, switch on air and set at 50-60, switch on fuel and set at 20 and lighting up frame?
9. Place sample tube in de-ionized water and set lamp/energy at 75.
10. Set SIGNAL to CONC, MODE to HOLD.
11. Key in: 3.00 t; 2.00 S1; 4.00 S2 and then press AZ.
12. Check prepared standards and confirm reading with S1,S2.
13. Measure and record calcium in samples.
14. Recalibrates the spectrophotometer every 10 to 15 samples.
15. When finish measurement, place the sample tube in de-ionized water. Switch off fuel, air, compressor, extractor fan. Turn GAIN and LAMP1 to zero. Switch off power. Turn off main acetylene.



# APPENDIX 4

## 4.1: HORMONAL LEVELS OF FISH NO. 833

| Spawning<br>cycle | Days | Total<br>calcium<br>(mg%) | Testosterone<br>(ng/ml) | Oestradiol-<br>17 $\beta$ (ng/ml) |
|-------------------|------|---------------------------|-------------------------|-----------------------------------|
| 1                 | 1    | 29.24                     | 7.14                    | 6.93                              |
| 1                 | 4    | 35.5                      | 13.44                   | 18.06                             |
| 1                 | 8    | 48.63                     | 45.36                   | 29.82                             |
| 1                 | 11   | 59.11                     | 76.44                   | 25.83                             |
| 2                 | 4    | 42.54                     | 8.19                    | 11.55                             |
| 2                 | 7    | 41.42                     | 21.84                   | 27.30                             |
| 2                 | 11   | 63.75                     | 48.30                   | 39.90                             |
| 2                 | 14   | 59.86                     | 46.20                   | 12.39                             |
| 2                 | 18   | 37.46                     | 10.08                   | 14.70                             |
| 2                 | 21   | 39.34                     | 22.26                   | 19.74                             |
| 2                 | 25   | 58.73                     | 57.96                   | 28.98                             |
| 2                 | 28   | 35.39                     | 63.0                    | 16.59                             |
| 2                 | 32   | 27.36                     | 11.42                   | 21.84                             |
| 2                 | 35   | 26.60                     | 28.98                   | 27.30                             |
| 2                 | 39   | 50.20                     | 39.48                   | 24.99                             |
| 2                 | 42   | 33.97                     | 2.1                     | 3.68                              |
| 2                 | 44   | 29.60                     | 3.90                    | 1.49                              |
| 3                 | 2    | 24.70                     | 3.36                    | 17.22                             |
| 3                 | 4    | 24.56                     | 8.4                     | 22.87                             |
| 3                 | 8    | 35.52                     | 15.12                   | 41.87                             |
| 3                 | 11   | 38.61                     | 36.75                   | 45.74                             |
| 3                 | 15   | 41.88                     | 37.80                   | 23.19                             |
| 4                 | 2    | 31.10                     | 2.90                    | 1.61                              |

#### 4.2: HORMONAL LEVELS OF FISH NO. 834

| Spawning<br>cycles | Days | Total<br>calcium<br>(mg%) | Testosterone<br>(ng/ml) | Oestradiol-17 $\beta$<br>(ng/ml) |
|--------------------|------|---------------------------|-------------------------|----------------------------------|
| 1                  | 1    | 34.01                     | 10.40                   | 9.24                             |
| 1                  | 4    | 35.42                     | 14.49                   | 18.06                            |
| 1                  | 8    | 53.19                     | 66.36                   | 32.55                            |
| 1                  | 11   | 49.70                     | 88.20                   | 22.26                            |
| 2                  | 4    | 34.64                     | 21.40                   | 19.53                            |
| 2                  | 7    | 46.25                     | 34.86                   | 48.30                            |
| 3                  | 1    | 60.11                     | 5.17                    | 6.83                             |
| 3                  | 5    | 40.41                     | 24.44                   | 24.78                            |
| 3                  | 8    | 69.53                     | 57.12                   | 25.20                            |
| 4                  | 1    | 60.18                     | 2.18                    | 12.18                            |
| 4                  | 5    | 41.60                     | 13.87                   | 4.41                             |
| 4                  | 8    | 45.56                     | 33.61                   | 17.64                            |
| 4                  | 12   | 49.20                     | 9.66                    | 4.00                             |
| 5                  | 3    | 23.82                     | 22.60                   | 18.90                            |
| 5                  | 7    | 40.02                     | 21.08                   | 16.38                            |
| 5                  | 10   | 48.36                     | 28.45                   | 3.28                             |
| 5                  | 14   | 28.90                     | 121.84                  | 21.26                            |
| 6                  | 1    | 24.89                     | 1.43                    | 1.87                             |
| 6                  | 5    | 37.21                     | 12.60                   | 16.43                            |
| 6                  | 8    | 33.97                     | 27.4                    | 41.23                            |



#### 4.3: HORMONAL LEVELS OF FISH NO. 970

| Spawning<br>cycles | Days | Total<br>calcium<br>(mg%) | Testosterone<br>(ng/ml) | Oestradiol-17 $\beta$<br>(ng/ml) |
|--------------------|------|---------------------------|-------------------------|----------------------------------|
| 1                  | 1    | 22.72                     | 11.76                   | 13.44                            |
| 1                  | 4    | 26.23                     | 75.60                   | 36.75                            |
| 1                  | 8    | 33.44                     | 78.30                   | 52.50                            |
| 1                  | 11   | 45.18                     | 84.60                   | 23.52                            |
| 2                  | 3    | 27.36                     | 24.36                   | 23.94                            |
| 2                  | 6    | 25.73                     | 44.10                   | 55.23                            |
| 2                  | 10   | 33.45                     | 57.96                   | 27.23                            |
| 2                  | 13   | 43.30                     | 42.84                   | 2.52                             |
| 2                  | 17   | 29.05                     | 15.12                   | 10.29                            |
| 2                  | 20   | 22.28                     | 6.30                    | 12.18                            |
| 2                  | 24   | 19.64                     | 8.60                    | 13.23                            |
| 2                  | 27   | 22.78                     | 15.96                   | 12.18                            |
| 2                  | 31   | 38.93                     | 19.32                   | 11.13                            |
| 2                  | 34   | 22.61                     | 25.54                   | 16.38                            |
| 2                  | 37   | 24.85                     | 9.16                    | 11.97                            |
| 3                  | 1    | 24.82                     | 6.22                    | 24.78                            |
| 3                  | 4    | 31.26                     | 8.82                    | 8.40                             |
| 3                  | 7    | 26.92                     | 24.49                   | 11.53                            |
| 3                  | 12   | 25.00                     | 37.72                   | 39.72                            |
| 3                  | 16   | 39.84                     | 54.86                   | 18.43                            |
| 4                  | 1    | 27.26                     | 7.63                    | 13.12                            |

#### 4.4: HORMONAL LEVELS OF FISH NO. 971

| Spawning<br>cycles | Days | Total<br>calcium<br>(mg%) | Testosterone<br>(ng/ml) | Oestradiol-17 $\beta$<br>(ng/ml) |
|--------------------|------|---------------------------|-------------------------|----------------------------------|
| 1                  | 1    | 21.96                     | 14.28                   | 11.86                            |
| 1                  | 4    | 27.55                     | 29.40                   | 15.42                            |
| 1                  | 8    | 34.01                     | 47.46                   | 28.78                            |
| 1                  | 11   | 35.64                     | 52.50                   | 20.75                            |
| 2                  | 3    | 29.43                     | 26.46                   | 21.34                            |
| 2                  | 6    | 22.84                     | 29.82                   | 19.87                            |
| 2                  | 10   | 43.61                     | 63.00                   | 24.32                            |
| 2                  | 13   | 40.10                     | 4.73                    | 8.79                             |
| 2                  | 17   | 31.38                     | 18.48                   | 12.45                            |
| 2                  | 20   | 34.83                     | 13.86                   | 15.76                            |
| 2                  | 24   | 24.60                     | 10.92                   | 28.52                            |
| 2                  | 27   | 21.02                     | 3.95                    | 32.13                            |
| 2                  | 31   | 38.39                     | 28.14                   | 15.79                            |
| 3                  | 2    | 57.12                     | 4.62                    | 39.30                            |
| 3                  | 6    | 33.68                     | 9.58                    | 48.31                            |
| 3                  | 9    | 29.05                     | 25.96                   | 1.29                             |
| 3                  | 12   | 37.35                     | 65.10                   | 47.03                            |



#### 4.5: HORMONAL LEVELS OF FISH NO. 972

| Spawning<br>cycles | Days | Total<br>calcium<br>(mg%) | Testosterone<br>(ng/ml) | Oestradiol-17 $\beta$<br>(ng/ml) |
|--------------------|------|---------------------------|-------------------------|----------------------------------|
| 1                  | 1    | 32.44                     | 7.98                    | 3.15                             |
| 1                  | 4    | 26.54                     | 31.16                   | 24.99                            |
| 1                  | 8    | 27.48                     | 40.32                   | 32.55                            |
| 1                  | 11   | 31.06                     | 62.58                   | 23.94                            |
| 1                  | 15   | 36.08                     | 6.30                    | 1.97                             |
| 1                  | 18   | 18.88                     | 6.72                    | 5.15                             |
| 1                  | 22   | 23.91                     | 31.08                   | 21.63                            |
| 1                  | 25   | 26.73                     | 6.72                    | 2.42                             |
| 1                  | 29   | 28.49                     | 28.56                   | 21.40                            |
| 1                  | 32   | 37.80                     | 37.80                   | 25.30                            |
| 1                  | 36   | 32.13                     | 20.51                   | 20.21                            |
| 1                  | 39   | 30.75                     | 16.80                   | 18.68                            |
| 1                  | 43   | 25.60                     | 10.20                   | 12.87                            |
| 2                  | 2    | 20.46                     | 7.56                    | 5.93                             |
| 2                  | 6    | 26.79                     | 12.60                   | 15.56                            |
| 2                  | 9    | 27.49                     | 24.36                   | 20.42                            |
| 2                  | 12   | 26.61                     | 30.24                   | 37.04                            |
| 2                  | 16   | 29.12                     | 11.13                   | 1.49                             |
| 3                  | 1    | 26.10                     | 1.30                    | 1.58                             |
| 3                  | 4    | 20.46                     | 3.36                    | 4.19                             |
| 3                  | 9    | 20.33                     | 15.12                   | 19.33                            |
| 3                  | 13   | 26.23                     | 12.91                   | 29.83                            |
| 3                  | 16   | 23.59                     | 39.90                   | 38.01                            |
| 3                  | 19   | 27.23                     | 48.76                   | 23.48                            |
| 3                  | 20   | 32.54                     | 53.23                   | 21.52                            |
| 3                  | 21   | 36.48                     | 58.34                   | 18.76                            |
| 4                  | 1    | 24.21                     | 7.62                    | 12.61                            |

#### 4.6: HORMONAL LEVELS OF FISH NO. 818

| Spawning cycles | Days | Total calcium (mg%) | Testosterone (ng/ml) | Oestradiol-17 $\beta$ (ng/ml) |
|-----------------|------|---------------------|----------------------|-------------------------------|
| 1               | 1    | 54.78               | 1.84                 | 1.38                          |
| 1               | 5    | 35.27               | 18.74                | 35.90                         |
| 1               | 8    | 36.02               | 17.60                | 39.06                         |
| 1               | 11   | 50.26               | 48.52                | 29.10                         |
| 1               | 14   | 57.23               | 3.31                 | 2.33                          |
| 2               | 1    | 47.69               | 0.95                 | 1.40                          |
| 2               | 3    | 33.82               | 10.89                | 20.92                         |
| 2               | 8    | 32.76               | 13.61                | 25.47                         |
| 2               | 12   | 38.91               | 30.14                | 26.99                         |
| 2               | 15   | 47.13               | 47.99                | 25.00                         |
| 3               | 1    | 33.76               | 0.76                 | 2.11                          |

#### 4.7: HORMONAL LEVELS OF FISH NO. 974

| Spawning cycles | Days | Total calcium (mg%) | Testosterone (ng/ml) | Oestradiol-17 $\beta$ (ng/ml) |
|-----------------|------|---------------------|----------------------|-------------------------------|
| 1               | 1    | 36.65               | 0.86                 | 3.23                          |
| 1               | 8    | 43.30               | 52.34                | 45.69                         |
| 1               | 12   | 43.93               | 49.58                | 25.10                         |
| 2               | 2    | 39.09               | 2.82                 | 1.98                          |
| 2               | 5    | 32.32               | 12.78                | 29.10                         |
| 2               | 8    | 47.31               | 34.53                | 33.68                         |
| 2               | 12   | 50.45               | 45.85                | 17.49                         |
| 2               | 15   | 29.30               | 7.26                 | 15.63                         |
| 2               | 19   | 33.01               | 28.01                | 33.40                         |
| 2               | 22   | 39.85               | 50.33                | 23.67                         |
| 2               | 26   | 33.57               | 62.82                | 23.20                         |
| 2               | 28   | 38.97               | 58.91                | 24.83                         |
| 3               | 1    | 26.48               | 5.88                 | 3.97                          |



#### 4.8: HORMONAL LEVELS OF FISH NO. 986

| Spawning<br>cycles | Days | Total<br>calcium<br>(mg%) | Testosterone<br>(ng/ml) | Oestradiol-17 $\beta$<br>(ng/ml) |
|--------------------|------|---------------------------|-------------------------|----------------------------------|
| 1                  | 1    | 24.97                     | 1.22                    | 0.97                             |
| 1                  | 8    | 30.31                     | 35.96                   | 46.84                            |
| 1                  | 12   | 45.18                     | 19.47                   | 13.78                            |
| 1                  | 14   | 38.72                     | 2.01                    | 3.22                             |
| 2                  | 8    | 39.03                     | 22.79                   | 32.60                            |
| 2                  | 11   | 40.73                     | 38.08                   | 29.57                            |
| 2                  | 14   | 43.29                     | 2.85                    | 4.22                             |
| 2                  | 25   | 31.94                     | 14.48                   | 14.47                            |
| 2                  | 28   | 33.07                     | 35.90                   | 30.06                            |
| 2                  | 29   | 34.39                     | 42.06                   | 22.75                            |
| 2                  | 30   | 34.14                     | 1.56                    | 0.72                             |

## APPENDIX 5

### 5: HORMONAL LEVELS OF *O. niloticus* FEMALES TREATED WITH LHRHa IMPLANTATION IN EXPERIMENT 4 (CHAPTER 7)

#### 5.1: Total calcium in plasma of the females in experiment4

| Days<br>post-<br>implant | Total calcium (mg%)  |                 |                              |                              |
|--------------------------|----------------------|-----------------|------------------------------|------------------------------|
|                          | Untreated<br>control | Sham<br>control | 75%cholest<br>erol<br>pellet | 95%cholest<br>erol<br>pellet |
| 0                        | 24.23±2.11           | 24.23±2.11      | 24.23±2.11                   | 24.23±2.11                   |
| 6 hrs                    | 26.08±2.46           | 22.49±0.79      | 24.54±1.09                   | 21.58±1.13                   |
| 1                        | 22.46±1.83           | 21.51±0.91      | 25.47±1.33                   | 21.07±1.23                   |
| 2                        | 22.34±1.21           | 20.85±1.21      | 22.35±1.26                   | 21.43±1.14                   |
| 6                        | 26.49±1.99           | 23.45±1.56      | 21.02±0.89                   | 23.93±1.13                   |
| 10                       | 24.95±2.12           | 22.37±1.77      | 24.95±1.14                   | 24.78±1.67                   |
| 13                       | 24.62±1.74           | 21.22±1.45      | 22.15±1.68                   | 23.07±1.22                   |
| 17                       | 20.59±1.07           | 22.60±1.45      | 22.59±1.53                   | 19.45±1.12                   |
| 20                       | 21.97±0.82           | 21.44±1.25      | 21.91±1.71                   | 22.14±1.94                   |
| 24                       | 22.71±1.86           | 22.20±1.39      | 21.60±1.62                   | 19.62±1.33                   |
| 27                       | 23.06±3.01           | 22.77±1.85      | 20.23±2.30                   | 19.80±1.79                   |
| 31                       | 22.33±2.28           | 23.99±1.87      | 21.41±2.39                   | 24.19±1.48                   |
| 34                       | 22.58±1.65           | 26.13±2.31      | 20.59±2.03                   | 24.57±1.49                   |
| 38                       | 22.09±2.23           | 20.80±1.76      | 25.23±2.63                   | 21.83±1.84                   |
| 41                       | 24.86±4.15           | 24.74±2.72      | 26.82±2.21                   | 23.84±1.96                   |
| 45                       | 23.85±2.21           | 28.04±2.37      | 28.29±2.37                   | 28.66±3.27                   |
| 48                       | 22.91±1.69           | 23.53±2.19      | 23.96±3.68                   | 22.38±1.21                   |
| 52                       | 24.15±2.03           | 24.57±2.63      | 25.11±1.80                   | 27.00±1.62                   |
| 55                       | 24.69±2.26           | 25.31±2.19      | 26.66±2.26                   | 26.43±2.22                   |



### 5.2: TESTOSTERONE IN PLASMA

| Days post-implant | Untreated control | Sham control | 75%cholest erol pellet  | 95%cholest erol pellet |
|-------------------|-------------------|--------------|-------------------------|------------------------|
| 0                 | 49.96±12.99       | 50.13±5.38   | 57.76±24.1 <sub>9</sub> | 49.49±10.2             |
| 6hrs              | 27.29±5.92        | -            | 48.03±10.1              | 55.79±11.6             |
| 1                 | -                 | 23.27±8.50   | 14.99±3.82              | 36.26±8.49             |
| 2                 | 48.16±10.84       | -            | 43.80±12.3              | 19.26±4.98             |
| 6                 | -                 | 31.33±9.75   | 26.26±4.05              | 23.09±5.75             |
| 10                | 27.38±11.79       | -            | 25.98±6.36              | 29.28±5.64             |
| 17                | -                 | 22.25±7.56   | 11.01±3.46              | 17.55±5.69             |
| 24                | 15.66±2.57        | -            | 9.96±2.22               | 7.47±2.28              |
| 31                | -                 | 30.79±9.04   | 21.70±5.05              | 21.92±5.77             |
| 38                | 25.82±5.15        | -            | 19.34±2.21              | 14.79±3.97             |
| 45                | 36.69±7.79        | 28.00±6.61   | 24.72±8.30              | 25.13±6.54             |
| 55                | 23.41±5.69        | 24.05±3.97   | 32.17±5.06              | 32.99±5.95             |

### 5.3: GONADOTROPINS (taGtH) IN PLASMA

| Days post-implant | Untreated control | Sham control | 75%cholest erol pellet | 95%cholest erol pellet |
|-------------------|-------------------|--------------|------------------------|------------------------|
| 0                 | 17.64±3.07        | 15.68±2.08   | 18.09±1.58             | 18.31±1.00             |
| 6 hrs             | 15.32±2.81        | -            | 78.05±25.0             | 74.98±21.3             |
| 1                 | -                 | 17.48±4.92   | 15.23±1.23             | 21.63±2.45             |
| 2                 | 12.08±1.39        | -            | 16.32±1.54             | 16.92±0.61             |
| 6                 | -                 | 18.13±3.28   | 14.91±1.12             | 21.98±5.12             |
| 10                | 16.38±3.50        | -            | 23.0±5.25              | 17.0±0.83              |
| 17                | -                 | 13.86±1.13   | 16.87±1.09             | 15.99±0.85             |
| 24                | 13.28±1.11        | -            | 12.60±1.50             | 16.89±3.05             |
| 31                | -                 | 13.17±1.87   | 19.32±2.93             | 17.56±0.86             |
| 38                | 10.16±1.31        | -            | 16.79±1.33             | 21.22±5.71             |
| 45                | 12.44±1.50        | -            | 65.99±20.6             | 23.63±5.63             |
| 55                | 10.10±3.60        | 15.95±0.74   | 17.61±0.93             | 17.11±2.39             |





